Title: The identification of tumour antigens recognized by patients with Duke's B (Stage II) reactive colorectal cancers using SEREX

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The identification of tumour antigens recognized by patients with Duke's B (Stage II) reactive colorectal cancers using SEREX

by

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ABSTRACT

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers in both men and women, posing a serious demographic and economic burden worldwide. In the UK, CRC affects one in every twenty people and it is often detected once well-established and after it has spread beyond the bowel (Stage IIA-C and Stage IIIA-C). A diagnosis at such advanced stages is associated with poor treatment response and survival. However, studies have identified two sub-groups of post-treatment CRC patients – those with good outcome (reactive disease) and those with poor outcome (non-reactive disease).

Evidence indicates the presence of an effective immune response differentiates between those patients who respond well to treatment and those who do not. To investigate these underlying mechanisms we used the serological analysis of cDNA recombinant libraries (SEREX) technique to determine which antigens are recognised by patients in each group. Immunoscreening a healthy donor testes cDNA library with sera from three patients with Duke's B reactive disease led to the identification of five antigens. These were **(1)** the immunoglobulin heavy constant gamma 3 (G3m marker), **IGHG3** gene, located on chromosome 14 at 14q32.33, which encodes IgG_3, and was recognised by sera from patients CC005 and CC014; **(2)** the immunoglobulin heavy constant gamma 2, **IGHG2** gene, located on chromosome 14 at 14q32.33 and recognised by CC014 sera; while **(3) CYB5R3, (4) RPL37A** and **(5) SLC34A2** were recognised by CC005, CC014 and CC014 sera respectively**.** CYB5R3 is a NADH-cytochrome b5 reductase 3 protein which has been shown to be upregulated in lung tissue with a RAS mutation in mice. Ribosomal protein L37a (RPL37A) has previously been shown to be upregulated in astrocytomas and to have a general association with lifetime glioblastoma survival and overall glioblastoma survival. Solute carrier family 34 member 2 (SLC34A2) encodes a protein which acts as a pH-sensitive sodium-dependent phosphate

transporter. SLC34A2 has been shown to be upregulated in breast and ovarian cancers and it is suggested that SLC34A2 is involved in the process of carcinogenesis, making it an attractive target in therapeutic strategies and also as a diagnostic biomarker. Although other antigens were found, and their sequences identified, all were unknown and not found in the databases.

RT-PCR analysis of the Duke's B colon cancer cell line SW480 showed consistent expression of **BCP-20.** Although, expression of SSX2, NY-ESO-1, TSP50, HAGE and RAGE were detected, the data was not easily reproductible. Further optimisation of the PCR conditions and primer pairs would be necessary to confirm these findings.

We hope in the future we can disern the role of these antigens in the inflammatory immune responses associated with reactive Dukes' B colon cancer which would help us better understand the mechanisms which underlie effective anti-tumour responses post-surgery. It may also be that RPL37A is a biomarker for patient survival in colorectal cancer and this would be worthy of further investigation.

DEDICATIONS

In memory of my beloved grandmother who always believed in me and inspired me to pursue my dreams no matter how impossible they seemed.

To Dr Barbara Guinn who is a true inspiration as a scientist, a role model and a friend. With a never-ending enthusiasm, positive energy and support she turned this research project into an unforgettable journey. Thank you for every moment.

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QUOTE

“Around here, however, we don’t look backwards for very long. We keep moving forward, opening up new doors and doing new things… and curiosity keeps leading us down new paths.”

- Walt Disney

PUBLICATIONS TO DATE

Peer-reviewed publications

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Conference presentations - Abstracts

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ABBREVIATIONS

AJCC	American Joint Committee on Cancer;
AML	acute myeloid leukaemia;
CEA	carcinoembryonic antigen;
CRC	colorectal cancer;
CTA	cancer testis antigen;
CTL	cytotoxic T lymphocyte;
DAC	5-aza-2'-deoxycytidine;
DC	Dendritic cells;
EBV	Epstein-Barr virus;
GIST	gastrointestinal stromal tumour;
IDP	intrinsically disordered protein;
pMHC	peptide MHC;
RAYS	recombinant antigen expression on yeast surface;
SADA	serum antibody detection array;
SEREX	**ser**ological identification of antigens by **re**combinant **ex**pression cloning;
SERPA	**SER**ological **P**roteome **A**nalysis;
SSX	synovial sarcoma X antigen;
SSX2	synovial sarcoma X breakpoint-2;
TAA	tumour-associated antigen;
TNM	Tumour Nodes Metastases;
Tregs	regulatory T cells;
TSP50	testes-specific protease 50.

CHAPTER 1 Introduction

1.1 The Global Challenge

'You have cancer' – the diagnosis that millions of people around the world hear every year and also the second leading cause of death worldwide. The number of new cancer cases each year is gradually increasing and the mortality rate is expected to rise from 7.6 million in 2008 to over 17 million in 2030 (Ferlay *et al*, 2010, Bray *et al*, 2012). On average, one of every three people is expected to experience some type of cancer in the course of their lifetime and this frequency is expected to increase (Quaglia *et al*, 2005) as the population ages. The World Health Organization (WHO) reports that an increasing percentage of all newly diagnosed cancer cases annually occurs in low- and middle-income countries and this is attributed to a wide range of behavioural, genetic and environmental risk factors (Thun *et al*, 2010). Nevertheless, the increase in the average life expectancy and the adoption of an unhealthy lifestyle (such as smoking, physical inactivity and poor diet) worldwide have also contributed to the rise of a new trend in the cancer demography. Newly-diagnosed cancers from the low- and middle-income countries today account for more than 51% of the total number and their share in the global burden is expected to continue to increase with the growth and aging of the population (Thun *et al*, 2010). In addition, the lack of resources for early cancer detection and effective treatments in the developing world contributes to an increase in cancer-related deaths.

Today, some of the most commonly diagnosed cancers both in economically developing and developed countries include prostate, breast, lung and CRC - "the big four" - accounting for nearly 50% of the total cases diagnosed (Jemal *et al*, 2010). Cancer is a global challenge, opposing a

serious demographic and economic burden with worldwide economic costs estimated to be as high as £572 billion per year. These alarming statistics resulted in the development of national strategies and action plans for cancer control including prevention, early detection and effective treatment (WHO Library Cataloguing-in-Publication data, 2008-2013). However, the demand for the development of new approaches to cancer screening and therapy is recognized globally and brings the research attention on these aims sharply into focus.

1.2 Colorectal Cancer (CRC)

CRC is one of the most commonly diagnosed cancers worldwide. It affects the bowel and the rectum and is rare in people under 40, with almost 85 per cent of cases being diagnosed in persons over 65 years of age (WHO Library Cataloguing-in-Publication data, 2008-2013). Statistics show that men and women are affected equally, while it is the third most common type of cancer in men (after prostate and lung cancer) and the second most common cancer in women (after breast cancer) (WHO Library Cataloguing-in-Publication data, 2008-2013). One in every twenty people in the UK develops CRC with only half of them surviving beyond five years, mainly because it is often detected once well-established and after it has spread beyond the bowel. The disease stage at the time of diagnosis governs both the choice of treatment and the prognosis. CRC is staged to reflect how far the cancer has spread and whether or not it has reached nearby structures such as lymph nodes or distant organs. The most commonly used staging system for CRC is that of the American Joint Committee on Cancer (AJCC), also known as TNM system (Edge *et al*, 2010). It describes three key pieces of information: '**T**' – how far has the primary **T**umour grown; '**N**' – the extent of spread to nearby lymph **N**odes; and '**M**' – describes whether the cancer has **M**etastasized. The

information from the **T**, **N** and **M** is combined to determine the cancer stage grouping from Stage I (the least advanced) to Stage IV (the most advanced). Two of the older staging systems include Duke's (Dukes, 1932) and Astler-Coller (Astler & Coller, 1954) but these are very rarely used today.

Stage I (A-C) (Duke's A-C) CRC is reported to be an asymptomatic malignancy, developing slowly by the progressive accumulation of genetic mutations within precancerous bowel lesions and polyps. Diagnosis at this stage reduces the risk of death from CRC, giving 90% chance of survival beyond five years (Mayer, 2005; O'Connell *et al*, 2004) and significantly low levels of disease recurrence. However, most cases of CRC are detected once the cancerous cells have moved beyond the middle layers of the colon (**Figure 1.1**). This is classified as stage IIB (Duke's B) and is one of the most commonly diagnosed forms of CRC. Currently, the course of treatment for CRC patients is fairly similar regardless of the significant differences in the biological features of each CRC case. Usually, the most effective approach is tumour resection, followed by chemo- or radiotherapy for stage III and sometimes for stage II CRC patients. However, recent studies suggest that not all stage III patients benefit from these therapies and that 25% of stage II cases are under treated (Marshall *et al*, 2007; Zaniboni *et al*, 2004). Furthermore, a number of non-aggressive tumours are frequently over-treated, leading to the patient experience of unnecessary and severe side effects. Methods such as, faecal occult blood test, sigmoidoscopy, colonoscopy, virtual colonoscopy and double contrast barium enema offer improvements in the detection rates of CRCs (Strul & Arber, 2007). However, their diagnostic value is limited with regards to costs, risks, lack of sensitivity especially in early stages and inconvenience to the patient (National Cancer Institute Factsheets). Therefore, the focus remains on developing efficient methods for the early detection of CRC such as the identification of early disease biomarkers which could be used in non-invasive (urine and blood serum) tests. Such

molecular biosensors for CRC would enable widespread screening alongside general health examinations and may further reduce the mortality rate associated with late stage detection of CRC (Kim *et al*, 2008).

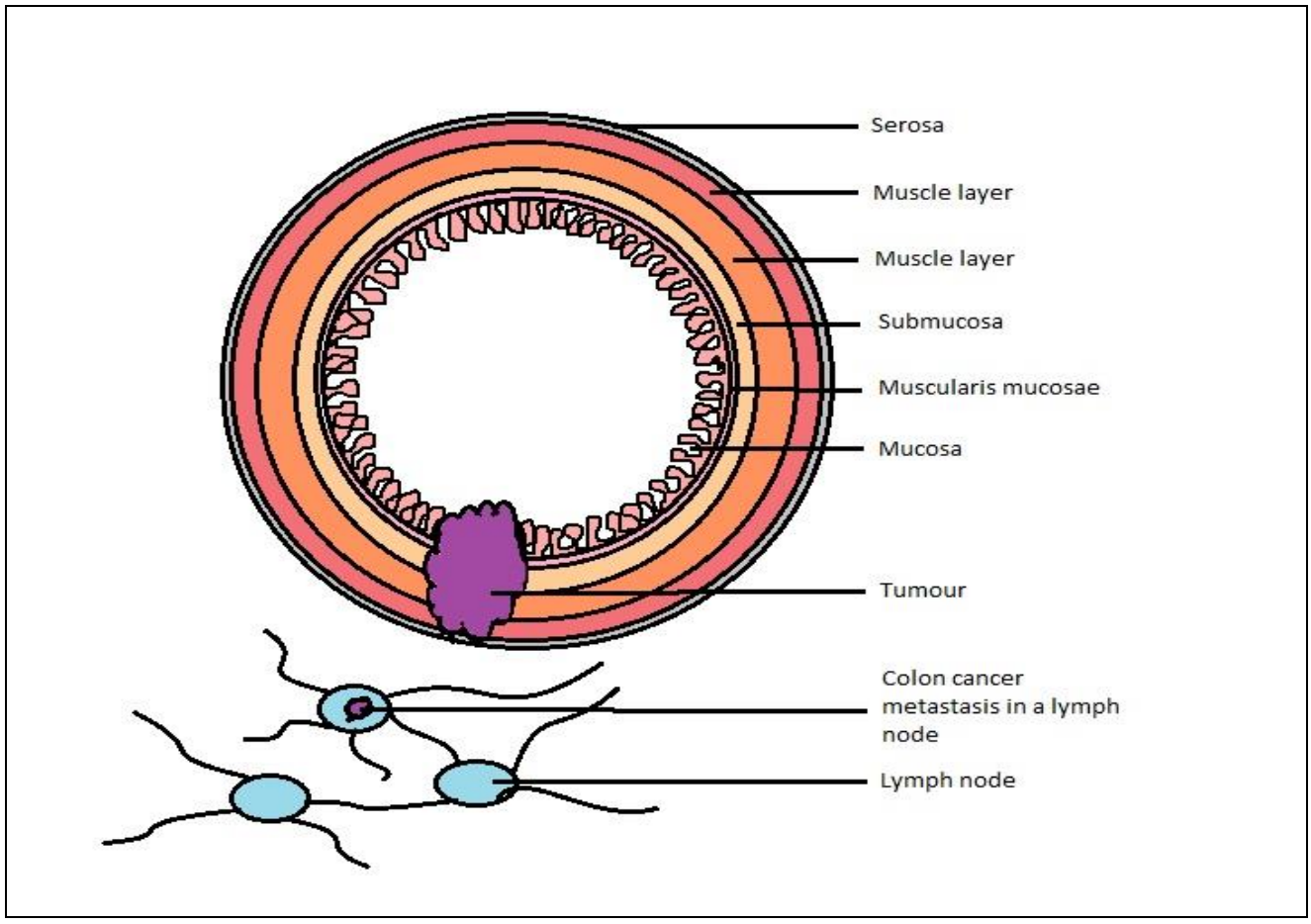


Figure 1.1 Diagrammatic representation of a malignant primary tumour that has moved beyond the middle layers of the colon. In this scenario the tumour cells have also metastasized to a nearby lymph node. This image indicates are stages of Duke's IIB to stage IV.

Murphy *et al*, 2000 suggested that there are two sub-groups of patients with Stage IIB CRC – those with good treatment outcome (reactive disease) and those with poor treatment outcomes (non-reactive disease). Whether the difference between these two groups of patients can be determined by differences in humoral responses warrants further investigation and provides the basis of our

own current studies. Tumour biomarkers offer an opportunity to translate unique CRC biological features into diagnostically pertinent information and would enable personalized treatments which could inform conventional and immunotherapeutic interventions. This would enable discerning treatment strategies for aggressive and non-aggressive cancers and the clear "up front" distinction of reactive from non-reactive disease.

In part, to address this need, researchers are investigating immune responses in cancer patients to identify new immunotherapy targets and biomarkers. They are hoping to identify and evaluate tumour-associated antigens (TAAs) and CTAs that could prove to be efficient diagnostic, prognostic or immunotherapeutic targets in CRC (Schlom *et al*, 2007). Recent developments in the fields of genomics and proteomics have greatly contributed to these studies, enabling the identification of multiple potential antigens within a single experiment (Wollscheid *et al*, 2004). Techniques, such as DNA microarray analysis, protein microarrays, peptide (pMHC) tetramers, **SE**rological identification of antigens by **R**ecombinant **EX**pression cloning (SEREX), **SER**ological **P**roteome **A**nalysis (SERPA) and peptide elution from MHC for mass spectrometry analysis are now commonly used to evaluate the expression profiles of genes and proteins as well as antigen recognition within different types, sub-types and stages of cancer. Their application in a number of studies conducted on myeloid leukemia (AML) (Guinn *et al*, 2005; Knights *et al*, 2006), diffuse large B-cell lymphoma (Liggins *et al*, 2004), lung cancer (Hanafusa *et al*, 2011) and osteosarcoma (Zou *et al*, 2012) have already proven successful and have led to the discovery of a large panel of antigens with prognostic and immunotherapeutic relevance. It has also provided an insight into the biological complexity and individuality of each cancer case, demonstrating considerable heterogenicity among patients and within tumour types. Therefore, genomics, proteomics or a combination of multiple methods could aid the discovery of novel biomarkers specific for CRC and

contribute to the development of personalized therapies which would maximize efficiency and minimize side effects for the patient.

1.3 The Search for Tumour Antigens Specific for CRC

The desire to identify TAAs has inspired and attracted researchers for more than four decades. Most efforts were driven by the aim to uncover specific epitopes on cancer cells which could elicit immune responses in the autologous host (Qiu & Hanash, 2009; Fratta *et al*, 2011). In the 1970s a method known as 'autologous typing' was used successfully to identify a number of antigens including alpha fetoprotein (in hepatoma and germ cell tumours), carcinoembryonic antigen (CEA) (in gastrointestinal cancers), prostate-specific antigen (in prostate cancer), CA125 (in ovarian cancer), and AU (in melanomas) (Old, 1981; Thomas & Sweep, 2001). This novel serological technique offered a substantial improvement over existing methods as it enabled the analysis of the cellular (T-cell defined) anti-tumour human immune responses in an autologous manner where only tumour-specific antigens could be recognized (Old, 1981). However, autologous typing did not completely fulfill the original hopes for its success due to several limitations. The major disadvantage arose from its reliance on cultured tumour cell lines and not all tumour types could be propagated *ex vivo* to allow autologous typing to be performed. Furthermore, only a small fraction of the total number of patients was found to have demonstrable levels of autologous antibody with specificity for cell surface antigens on their tumour (Qiu & Hanash, 2009; Fratta *et al*, 2011). In the cases when the technique was successful and a tumour antigen was identified, the low titer of antibodies that were often detected made any further biochemical or molecular characterization of the antigens almost impossible. However, the method of autologous typing contributed to the

identification of a number of human tumour antigens which were categorized into four classes: *Differentiation antigens* – e.g. gp-100; *Mutational antigens* – e.g. abnormal forms of p53; *Retroviral antigens* – Epstein Barr Virus (EBV) and Human Papilloma Virus (HPV); and *CT antigens* (Fratta *et al*, 2011).

1.3.1 CT Antigens

CTAs were classified as a type of TAAs with restricted expression in testis, placenta and various types of cancer (Fratta *et al*, 2011). In the field of cancer serology they were quickly identified as the ideal targets for tumour-specific immunotherapeutic approaches (Simpson *et al*, 2005; Lim *et al*, 2012; Caballero *et al*, 2009; Scanlan *et al*, 2002; Cheng *et al*, 2011).

The selective expression pattern of CTAs is considered to be a consequence of gene activation by DNA demethylation (De Smet *et al*, 1995; Ogawa *et al*, 2005) and histone post-translational modifications (Karpf, 2006) both occurring constitutively in the testis, but also in tumour cells (Ogawa *et al*, 2005). Some CTAs have been found to be expressed in healthy tissues such as liver, pancreas and spleen but at a significantly lower level (<1%) when compared to their expression in testis (Caballero & Chen, 2009). Around 70 families of CTAs have been identified to date and further classified as X-CTAs and non-X-CTAs depending on the chromosomal location to which the genes are mapped (Old, 2001). The genes for distinct X-CTAs have been previously reported to encode for different antigenic peptides that are presented with HLA class I or HLA class II allo-specificities, eliciting both cell-mediated and humoral immune responses (Traversari, 1999). The blood-testis barrier and the lack of HLA class I expression on the surface of germ cells prevents the cells of the immune system from interacting with the CTAs expressed there (Medin, 2010). It has been suggested that as the B and T cells have not been previously challenged by CTAs the immune

response should be able to recognize CTAs as non-self structures when expressed on cancer cells (Medin, 2010) circumventing the need to break tolerance. These findings suggest that CTAs can be viewed as promising molecular targets for the development of immunotherapeutic interventions for specific cancer types without the possibility of triggering autoimmune responses. The identification of CTAs specific for a particular type of cancer is a promising approach for the development of peptide or recombinant full-length protein anti-cancer vaccines and for antigen-specific adoptive T-cell transfer as part of cancer therapy (Slingluff, 2011). In that respect, NY-ESO-1 (a CTA with strong immunogenicity still detectable at sera dilutions of 1 in 40,000 (Jager *et al*, 1998)) has been of interest in relation to the development of cancer vaccine trials. Several ongoing trials have been based on this antigen alone or its use in combination with an immune enhancer are currently ongoing (Roswell Park Cancer Institute, 2012; Cancer Research Institute, 2009). However, the expression of other CTAs within various types of cancer and at different disease stages appears to be heterogeneous and further investigation is required for the identification of effective immunotherapeutic targets.

Although, CTA expression is generally correlated with tumour progression and immunogenicity in various types of cancer, little is known about this in relation to CRC. In addition, CTAs were often found to be poorly expressed (low level, heterogeneous expression) in CRC (Marits *et al*, 2009) highlighting the need for the identification of further immunogenic CTAs involved in CRC development. Such proteins would be candidates for cancer-specific therapy trials and may provide effective biomarkers for diagnosis, prognosis and monitoring of CRC disease progression.

1.3.2 SEREX

The limited success of autologous typing demonstrated the need for a more comprehensive experimental approach for the identification of TAAs. It was not until the mid-1990s when a new autologous immunoscreening technique was shown to circumvent some of the limitations of autologous typing. This method was called SEREX (Sahin *et al*, 1995). It allowed the identification of numerous TAAs based on their recognition by antibodies in diluted pre-cleared autologous patient sera and in a small number of experiments. The advantages of SEREX were quickly recognized and a substantial pool of data encompassing serologically relevant antigens within cancer began to collect. SEREX was capable of identifying immunoglobulin G (IgG) antibody responses to both highly (NY-ESO-1) and weakly immunogenic TAAs (Chen *et al*, 1997; Old & Chen, 1998). Approximately one-third of these antigens were novel and referred to as SEREX-defined antigens. In addition, sera was shown to be able to detect $CD8^+$ T-cell recognized TAAs including NY-ESO-1 (Jager *et al*, 1998), MAGE-1 and tyrosinase (Jager *et al*, 1998; Sahin *et al*, 1995; Tureci *et al*, 1997) as well as TAAs recognized by IgG antibodies which are also known to require $CD4^+$ T-cell help (NY-ESO-1) (Sahin *et al*, 1995; Chen *et al*, 1997; Tureci *et al*, 1997; Zeng *et al*, 2001; Nakatsura *et al*, 2001; Nishikawa *et al*, 2001). These findings demonstrate that the cellular and humoral immune system work in concert and are both stimulated by TAAs in the case of cancer. Furthermore, several antigens (NY-ESO-1, MAGE-1 and hMena) (Modugno *et al*, 2004) that elicit humoral as well as cell mediated immune responses have also been successfully identified through SEREX. Studies have investigated $CD8^+$ and $CD4^+$ T-cell recognition of SEREX-defined antigens and the data showed that co-immunization with such proteins in combination with a cytotoxic T lymphocyte (CTL) epitope enhanced $CD8^+$ induction in a $CD4^+$ T cell–dependent manner (Nishikawa *et al*, 2005; Neumann *et al*, 2005).

The continuously increasing number of SEREX antigens led to the creation of a Cancer Immunome Database, a free repository for the antigens identified by this serological approach (Cancer Immunome Database). The success of SEREX lies in its reliance on the construction of a cDNA library from human tissues (cell lines, primary tumour or normal donor testes) and their expression in a prokaryotic system (Sahin *et al*, 1995). Immunoscreening with SEREX permits quick and effective extraction, processing, sequencing and subsequent molecular analysis of the protein of interest as both the TAA and its coding cDNA are present in the same plaque. However, the use of SEREX has several limitations. It has been previously reported that SEREX-defined antigens are predominantly nuclear proteins which are often transcription factors (Jager *et al*, 1998) and/or ubiquitously expressed (Cancer Immunome Database; Hartmann *et al*, 2005). As such, they rarely show evidence of mutations or other structural abnormalities and are often weakly immunogenic, incapable of eliciting and sustaining strong humoral immune responses (Qiu & Hanash, 2009). In addition phage can only express proteins in their primary structure which may cause the failure to detect antigenic sequences consequent to eukaryotic post-translational modifications (Mischo *et al*, 2003). Regardless of the limitations, SEREX-defined antigens are already being investigated as diagnostic markers including Testis-Specific Protease (TSP50) in ovarian and CRCs (Shan *et al*, 2002)) and as immunotherapeutic targets such as OVA66 in ovarian cancer (Jin *et al*, 2008), KIF20A in pancreatic cancer (Imai *et al*, 2011).

1.3.3 SERPA

SERPA is a powerful tool used for the identification and validation of immunogenic TAAs. Similar to SEREX, it uses the antibody repertoire contained within the sera of a cancer patient to successfully identify TAAs (Klein-Scory *et al*, 2012). In comparison to SEREX, SERPA does not require the use of a cDNA library, making this method less time consuming and less labour

intensive. Furthermore, SERPA is better suited for the detection of possible post translational modification and protein isoforms as it relies on the separation of complex mixtures of proteins extracted from cell cultures or tumours. The separation is performed via the use of a two dimensional gel electrophoresis (2-DE) (Forgber *et al*, 2009a). A number of TAAs have been identified to date using SERPA in various types of cancer including lymphoma (Forgber *et al*, 2009b), renal cell carcinoma (Klade *et al*, 2001), ovarian cancer (Gagnon *et al*, 2008) and CRC (He *et al*, 2007). These studies have established the specificity of SERPA and its great potential in uncovering immunogenic TAAs and identifying tumour markers. However, similar to SEREX, most of the antigens identified by SERPA are predominantly weakly immunogenic intracellular proteins and very rarely membrane-associated ones (Suzuki *et al*, 2010; Chen, 2004). This and the finding that a number of antigens found by T cell cloning have also been found by SEREX suggests that most antigens induce B and T cell responses (Jager & Knuth, 2004), although not necessarily to the same epitope(s). In addition, SERPA has demonstrated a drawback associated with the use of 2-DE: low abundance (such as regulatory and signal transduction proteins and receptors (Chevalier, 2010)), hydrophobic or insoluble proteins (such as membrane proteins (Chevalier, 2010)) are inherently difficult to detect (Suzuki *et al*, 2010).

SEREX and SERPA are two methodologies that seem complementary to each other as they identify two different sets of antigens (SEREX for the identification of antigens with altered expression; SERPA for the identification of antigenic proteins resulting from post-translational modifications). Therefore, the identification and validation of CRC-related CTAs using SERPA and SEREX independently or as a combined approach is worthy of further consideration.

1.3.4 RAYS

RAYS is another serological strategy applied to the process of discovering immunogenic CTAs. RAYS permits the expression of immunogenic proteins on the surface of yeast allowing for a more natural folding of the protein and partial glycosylation (by virtue of it being a eukaryotic system) (Mischo *et al*, 2003). This permits the analysis of proteins in their natural conformation when compared to the prokaryotic expression utilized by SEREX. This method has demonstrated specificity and sensitivity for the detection of an antibody response to a conformation-dependent epitope - the colorectal cancer antigen A33. Therefore, the CTAs that have escaped detection due to the fact that they elicit immune responses only after undergoing the appropriate post-translational modifications could potentially be identified via RAYS. To date, RAYS has allowed the confirmation of antigen immunogenicity through screening of eukaryotic cDNA expression libraries derived from pancreatic cancer (Wadle *et al*, 2006) and prostate cancer (Jung *et al*, 2010). RAYS offers a less time-consuming analysis of the serological autoreactivity in cancer patients and it has provided an effective anticancer vaccine platform (recognizing NY-ESO-1) in prostate cancer patients (Jung *et al*, 2010). However, its further development is required to allow the detection of novel target antigens (such as is achieved with SEREX or SERPA) and its application in CRC requires further analysis.

1.3.5 SADA

To evaluate the seroreactivity of a number (or a panel) of SEREX-defined antigens in a particular type of cancer, a spot immunoassay, known as SADA, has been successfully developed and utilised (Scanlan *et al*, 2002). Although, several antigens have been evaluated in cases of colon cancer

(MAGEA3, SSX2, and NY-ESO-1 (Scanlan *et al*, 2002)), additional improvements in the sensitivity of SADA are still required.

1.3.6 Protein microarrays

Protein microarrays allow the rapid and easy detection of tumour antigens using patient sera (Stempfer *et al*, 2010; Gunawardana & Diamandis, 2007). Recent studies have demonstrated the efficiency of the technique in the rapid identification of immunogenic membrane-based TAAs with high reproducibility of the experimental analyses of lung and brain (Stempfer *et al*, 2010) and ovarian (Gunawardana & Diamandis, 2008) cancers. In this context, protein microarrays has a great advantage as this methodology allows the construction and simultaneous analysis of a large panel of candidate tumour biomarkers (approximately 9,000). However it should be noted that although protein microarrays have vast screening potential they are limited to defined proteins from a limited albeit not insubstantive pool.

Understanding the molecular interactions between T cell receptors on cytotoxic T lymphocytes (CTL) and peptide/MHC class I complexes on tumour cells is an essential tool for the development of immunogenic vaccines (Tsukahara *et al*, 2008). Several such vaccines have already been designed based on TAA epitopes and have been implemented in phase I and II clinical trials for different types of cancer (HPV (Muderpach *et al*, 2000), WT1 (Oka *et al*, 2004), human telomerase reverse transcriptase (Bolonaki *et al*, 2007) and HLA-A24^{+}HRPC (Noguchi *et al*, 2005) peptide vaccinations). The strength of vaccine-mediated immunological responses generally need to be enhanced and this will be much more feasible in patients in subsequent (Phase III) clinical trials who are likely to have less advanced disease. However the results obtained from clinical trials to date warrants further investigation.

1.4 Tumour profiling in patients with CRC

A number of studies have been conducted over the last decade aiming to identify novel biomarkers that would prove to be efficient in CRC profiling as prognostic, predictive or therapeutic biomarkers. The recent improvements in proteomics and genomics methods have greatly aided this aim and have led to the identification of a number of CTAs and TAAs relevant to CRC. However, the clinical significance of only a small fraction of these potential markers in CRC has been evaluated to date:

1.4.1 CTAs in CRC

NY-ESO-1 has been recently studied in relation to CRC. It was demonstrated that some CTAs are capable of eliciting strong humoral and cell-mediated immune responses in some patients with CRC (Stockert *et al*, 1998). However, its expression in CRC is often highly heterogeneous, when present, which poses an obstacle in the development of a generalized immunotherapy. As discussed earlier in this review, NY-ESO-1 has also been targeted in several vaccine clinical trials worldwide involving CRC patients. Recently, a new Phase I trial of a fusion protein vaccine is being organized targeting solid tumours expressing NY-ESO-1 and this investigation includes Stage I – IV CRC (U.S. National Institute of Health, 2012). In addition, NY-ESO-1 expression was shown to correlate with CRC stages and local lymph node metastasis (Li *et al*, 2005) making it a potential prognostic biomarker for CRC.

TSP50 was originally identified as abnormally expressed in breast cancer cells (Yuan *et al*, 1999) and has recently been studied for the first time in CRC patient samples. The expression of TSP50 was found to correlate with the clinicopathological characteristics and disease-specific survival for CRC patients (Zheng *et al*, 2011). Furthermore, the study demonstrated that TSP50 expression is

highly specific for CRC as compared to colorectal adenomas and normal tissues, allowing the easy differentiation between them. TSP50 is an attractive predictive biomarker for poor survival in patients with early stages CRC (Stage I and II), but not in patients with advanced stage disease. As such, it is the only effective predictive biomarker reported to date for patients with early stage CRCs (Zheng *et al*, 2011).

CABYR is a calcium-binding tyrosine phosphorylation-regulated fibrous sheath protein and its expression was first identified in human spermatozoa (Naaby-Hanen *et al*, 2002; reviewed in Chiriva-Internati *et al*, 2008). Subsequent detection of CABYR in lung carcinoma and its absence in healthy tissues, led it to be considered as a novel CTA, which has been shown to have some immunogenic properties that could serve as a basis for the development of immunotherapy for cancer patients (Luo *et al*, 2007). Additional studies on CABYR expression in brain (Hsu *et al*, 2005), hepatocellular (Li *et al*, 2012) and other carcinomas (Tseng *et al*, 2011) have shown that there are at least five different isoforms of this protein which could play unique roles in the process of carcinogenesis. Following these discoveries a recent study has reported a frequent overexpression of CABYR a/b and c isoforms in CRC tumours when compared to adjacent normal tissues (Shantha Kumara *et al*, 2012). However, a more comprehensive investigation is required to determine whether CABYR expression correlates with tumour stages and is a suitable therapeutic vaccine candidate in CRC.

SPAG9 is another antigen found to be exclusively expressed in testis (Jagadish *et al*, 2005) that is a particularly attractive target for immunotherapy in epithelial ovarian cancer (Garg *et al*, 2007), thyroid cancer (Garg *et al*, 2009) and in CML (Kanojia *et al*, 2010). A recent study has investigated the expression of SPAG9 in CRC patients aiming to explore its possible role in colon cancer tumorigenesis and its effectiveness in eliciting a humoral immune response (Kanojia *et al*, 2011).

Interestingly, the study has reported a close relationship between SPAG9 expression and early stages of CRC development suggesting that it could serve as an early diagnostic biomarker for CRC patients. The investigation had also demonstrated that SPAG9 could play a key role in the tumour development and could also serve as a target for the development of immunotherapeutic methods.

Other CTAs, found to exhibit a strong correlation with CRC presentation are listed in **Table 1.1.**

1.4.2 TAAs in CRC

CD133 is a cell surface protein marker found on undifferentiated cancer cells that exhibit stem-like properties. These cells account for the propagation, growth and recurrence of AML (Lapidot *et al*, 1994; Krivtsov *et al*, 2006) and CRC (Wang *et al*, 2012; Ricci-Vitiani *et al*, 2007) and for the resistance of these cancers to current therapies. The functional importance of CD133 expression has been investigated in several studies in relation to the initiation and behaviour of CRC (Ricci-Vitiani *et al*, 2007; Horst *et al*, 2009a; Choi *et al*, 2007). The $CD133^{+}$ CRC stem cells are reported to have exhibited the ability to transfer cancer to a secondary recipient maintaining the same immunophenotype and the global gene expression profile of the primary tumour when compared to $CD133^{-}$ cells (Ricci-Vitiani *et al*, 2007; Horst *et al*, 2009a). Furthermore, several studies have clearly identified the correlation between CD133 expression alone (Horst *et al*, 2009a; Choi *et al*, 2007) or in combination with other protein markers (Horst *et al*, 2009a) with CRC patient survival and have revealed it to be a reliable prognostic marker. In combination with CD44 and CD166 (cell surface protein markers), CD133 expression has also been linked to the presentation of low-, intermediate-, or high-risk CRC cases with the ability to distinguish between them at an early stage of the disease (stage II) (Horst *et al*, 2009a; Horst *et al*, 2009b). CD133 is a promising predictive and prognostic marker in the diagnosis of CRC and particularly of interest as it is applicable to the

early stages of the disease. However, the presence of such cell surface markers on the CRC stem cells has not been investigated in relation to the underlying cause of the non-reactive type of CRC.

Table 1.1 Antigens shown to be potential diagnostic, prognostic or immunotherapeutic targets in CRC.

CTA/ panel of CTAs	Chromosome location	Method of identification	First identified in:	Potential uses in CRC
BCP-20 (FBXO39)	17p13.1	SEREX	CRC (Song *et al*, 2011).	Candidate diagnostic and immunotherapeutic target (Song *et al*, 2011).
PAGE4 **SCP-1** **SPANX**	Xp11.23 1p13-p12 Xq27.1	Database mining SEREX Differential display	CRC (Chen *et al*, 2010).	Predictive panel for liver metastasis (Chen *et al*, 2010).
MAGE-A4	Xq28	T cell epitope cloning	Melanoma (De Plaen *et al*, 1999).	Colon cancer vaccine therapy with peptide of MAGE-A4 (Takahashi *et al*, 2012).
STK31	7p15.3	Three-step Microarray Analysis	CRC (Yokoe *et al*, 2008).	Candidate target for immunotherapy (Yokoe *et al*, 2008).
SSX **MAGE**	Xp11.2 Xq28	Reverse transcription-PCR	CRC (Choi *et al*, 2012).	Co-expression as predictive marker for metastasis. Candidate targets for immunotherapy (Choi *et al*, 2012).
SSX2	Xp11.22	SEREX	Melanoma (Tureci *et al*, 1996).	Candidate target for immune therapy (Ayyoub *et al*, 2004; Smith *et al*, 2010).

Carcinoembryonic antigen (CEA) is a TAA whose expression levels are often monitored pre- and post-treatment in CRC patients as they have been found to be indicative of cancer recurrence (Lin *et al*, 2011) and poor disease prognosis (Yeh *et al*, 2011; Kira *et al*, 2012). Patients with elevated post-treatment CEA expression levels are often monitored more carefully for relapse of CRC and for local or distant recurrence (Lin *et al*, 2011; Kirat *et al*, 2012). To date, several studies have investigated the potential of CEA as an immunotherapeutic target in cases of CRC. Different research strategies have incorporated CEA peptides or CEA mRNAs in dendritic cell (DC) vaccines (Burgdorf, 2010; Lesterhuis *et al*, 2010) and in plasmid DNA vaccines (Staff *et al*, 2011) demonstrating that these vaccines are well-tolerated and have immune-stimulatory capacity in patients with CRC. However, the overall outcome of these studies indicated that additional vaccine modulation is necessary to attain significant clinical impact. More recently, a study to investigate whether the vaccination of toll-like receptor activated DCs can induce more potent CTL responses and antitumour activity in CEA transgenic mouse tumour models was published (Hong *et al*, 2012). It has demonstrated that the combined activation of TLRs can lead to better maturation status of DCs and can also induce more effective antitumour immune responses against CRC. However, additional investigation is necessary to evaluate the effectiveness of this approach in human models.

1.4.3 Clinical significance of CTAs in CRC

The CTAs that have already been identified within different types of cancer could serve as biomarkers for discerning aggressive and non-aggressive cancers and for predicting treatment outcome and relapse. Their expression patterns and clinical significance is still under investigation, but there are very promising early results. Some of their clinical applications are described in the followings sections, and could expand the list of potential CTAs in CRC.

1.5 Potential biomarkers for discerning reactive from non-reactive disease

The expression profile of CTAs in relation to treatment outcome in particular types of solid cancers has been previously studied on several occasions (Zou *et al*, 2012; Modugno *et al*, 2004; Shan *et al*, 2002; Jin *et al*, 2008; Imai *et al*, 2011) but further investigation is necessary to compare these findings to CRC cases. In this context, a recent study has demonstrated that γ-irradiation *de novo* up-regulates the expression of various CTAs and MHC-I in a randomized fashion. Therefore, irradiation could be accounted responsible for the increased immunological response to certain tumours, due to the inflammation and cell damage it causes. This would be anticipated to cause the immune system to attend the site of damage, mop up cellular debris and present proteins including CTAs to the immune system (Sharma *et al*, 2011). These findings fit with demonstrations in leukaemia that elevated tumour antigen expression at disease presentation is associated with improved survival (Guinn *et al*, 2009; Greiner *et al*, 2008). Identifying and evaluating such TAA and CTAs would be beneficial for profiling individual tumours and for combining radiotherapy (or other cancer therapy approach) with immunization to maximize the effect of treatment in CRC. However, in order to design a combined treatment a thorough understanding about the mechanisms of initiating CTAs expression and the likely order of their expression is necessary.

The exact role of the numerous CTAs in relation to tumour response to various treatments still remains poorly defined. Particularly, in cases where adjuvant therapy is in order, it would be beneficial to have a panel of biomarkers to predict the likely success of the therapy. Several CTAs have been evaluated in gastrointestinal stromal tumour (GIST) – with regards to recurrence, while levels of MAGE-A1, MAGE-A3, MAGE-A4, MAGE-C1 and NY-ESO-1 expression were investigated in response to imatinib adjuvant therapy (Perez *et al*, 2011). This study demonstrated that CTA^+ GISTs had a significantly shorter recurrence free survival compared to negative case.

Furthermore, the expression of NY-ESO-1 and MAGE-A3 was associated with elevated resistance to imatinib and therefore, with continuous tumour progression. Luetkens *et al*. have also showed that PRAME expression remains stable under imatinib treatment and correlates with decreased overall survival in patients with CML (Luetkens *et al*, 2010).

Similar findings have been reported in several studies of prostate and lung cancer, multiple myeloma, AML, liposarcoma and others (Smith *et al*, 2011; Shiraishi *et al*, 2011; Boehmer *et al*, 2011; Tajima *et al*, 2003; van Duin *et al*, 2011; Pollack *et al*, 2012). These suggest an important role of CTAs in the pathophysiological behaviour of different tumours in response to treatments. Further investigation of panel of antigens associated with particular types of cancer and their relationship to either reactive or non-reactive disease is yet to be attempted. In this relation, TSP50 is the only known CTA to have been characterized as a biomarker for disease prognosis in CRC (Zheng *et al*, 2011), but its association with treatment outcome is still to be analyzed. Furthermore, investigation of the interrelationships between groups of CTAs in CRC and their expression profiles could lead to significant discoveries about the underlying cause of a particular treatment response. This could benefit the design of a multivalent cancer vaccine targeting several antigens rather than just a single one.

1.6 Potential biomarkers for survival prognosis

Several studies have investigated the role of the CTAs as biomarkers of prognostic value regarding patient survival. Elevated levels of expression of particular CTAs have been shown to correlate with poor survival prognosis, particularly in solid tumours. In contrast to expectations some antigens have been shown to have above average levels and have better survival rates in patients with

haematological malignancies. For example, expression of SSX2IP in the presentation of AML has been shown to predict good survival in patients with no detectable cytogenetic rearrangements (Guinn *et al*, 2009). The elevated levels of antigens provide targets for improved immune responses in patients post-conventional (chemotherapy, radiotherapy) treatment, when there is cancer cell damage and inflammation (danger) signals stimulating an immune response to clear up dead and dying cells. Furthermore, the CTAs expressed on non-solid tumours (such as AML) are more accessible and "easier to see" by the immune system as they are not hidden within heterogeneous layers of cancer cells (as seen in solid tumours).

The prognostic value of CTAs has also been evaluated in the presentation of osteosarcoma by gene microarray where the high expression of MAGE-A could predict distant metastasis and poor survival. For patients with and without MAGE-A expressing tumours, the five-year survival rates were found to be 39.6% and 80% respectively (Zou *et al*, 2012). Similarly, increased levels of WT1 (another CTA) expression have been shown to correlate to poor prognosis and relapse in paediatric acute myeloid leukaemia after induction therapy (Lapillone *et al*, 2006). A number of products of translocations have been used in routine labs which detect minimal residual disease and can indicate impending relapse with high accuracy (John *et al*, 2002). The CTA expression in relation to patient survival in CRC cases has been the focus of few studies and further investigation is required to establish the clinical significance of this relationship (Zheng *et al*, 2011).

1.7 Potential biomarkers for discerning aggressive from non-aggressive disease

A recent study on prostate cancer has demonstrated that several CTAs are preferentially expressed in either aggressive or non-aggressive disease (Shiraishi *et al*, 2012). Such biomarkers could be

particularly useful in preventing the overdetection and overtreatment of potentially indolent CRC or the undertreatment of a more aggressive type of disease.

1.8 Potential immunotherapeutic targets

In 2005, cancer patients expressing NY-ESO-1 and LAGE-1 antigens entered a Phase I clinical trial on a plasmid DNA (pPJV7611) cancer vaccine (Gnjatic *et al*, 2009), demonstrating the effectiveness of CTAs as immunotherapeutic targets. Recently, the first clinical trial of cancer vaccine therapy with artificially synthesized helper/killer-hybrid epitope long peptide of MAGE-A4 cancer antigen was initiated (Takahashi *et al*, 2012). A patient with pulmonary metastasis of CRC was vaccinated and had shown a significant reduction in the tumour growth.

1.9 Non-reactive disease – overcoming the underlying issues

CTAs are key molecules in the field of cancer serology. Their progressively expanding family is continuously providing potential novel targets for cancer immunotherapy or diagnostic/prognostic examinations. However, CTAs have also been evaluated for their role in oncogenesis, particularly their contribution to the immortality, invasiveness, immune evasion, hypomethylation and metastatic capacity of the neoplasms (Zhang *et al*, 2010). Investigating the correlation between CTAs and the clinical presentation of CRC (reactive or non-reactive type) requires knowledge of the molecular mechanisms that govern their expression and physiological functions - mechanisms that still remain poorly understood. According to recent studies, genome wide hypomethylation is accounted for the aberrant expression of CTAs within CRC cells (Mossman *et al*, 2010; Robins &

Rees, 2001; Glazer *et al*, 2009). However, the epigenetic factors that dictate which panel of silenced genes to be reactivated and the physiological properties of these particular CTAs are still unknown. This is an area of actively ongoing research as it is believed that these factors are responsible for the heterogenicity in the CTA expression profiles of the individual CRC cases. A better understanding of the structural and serological properties of these antigens, their modes of expression and functions, should establish whether they are the ones governing the reactive and non-reactive CRC phenotypes.

CTAs in CRC have been associated with low immunogenicity under normal conditions, and only a few patients actually recognise the peptide epitopes and exhibit strong CTL or humoral immune response to CTAs. This could be partially accounted to the structural stability of the proteins, encoded by the CT genes. It has been previously demonstrated that proteins must be cleaved to small peptides by intracellular proteinases upon presentation to the immune system (Goldberg & Rock, 1992). However, high stability proteins (α-helical secondary protein conformation known to provide the most optimal structural stability (Robins & Rees, 2001)) are less likely to undergo denaturation and polypeptide chain unfolding. Therefore, CTAs containing large proportions of α-secondary structure should be more resistant to protein unfolding and subsequent cleavage by the proteinases, thus resulting in an unsuccessful presentation to CTLs (low immunogenicity). For example, SCP-1 (a CTA of particularly low immunogenicity) has been shown to contain 76.6% α-helical structure from its total secondary conformation when compared to the highly immunogenic NY-ESO-1 CTA, containing only 20.7% (Robins & Rees, 2001). The identification of such antigens that are predominantly expressed in non-reactive CRC patients could potentially be accounted for the therapy-resistant presentation of the disease. In such cases epigenetic modulation to induce expression of other CTAs may highly favour the immunotherapeutic approach to non-

reactive CRC disease. In this respect, a study has recently reported a successful induction of NY-ESO-1 expression in CRC cells (but not in normal nontransformed cells) both *in vitro* and *in vivo* subject to hypomethylating agent 5-aza-2'-deoxycytidine (DAC) treatment (Chou *et al*, 2012). The study reports that DAC-treated CRC cells are susceptible to major histocompatibility complex (MHC)-restricted recognition by $CD8^+$ NY-ESO-1-specific T cells. A NY-ESO-$1_{157-165}$-specific T-cell receptor was successfully used to generate both $CD8^+$ and $CD4^+$ NY-ESO-$1_{157-165}$-specific T cells that selectively recognized DAC-treated CRC cells but not non-treated cells. These data reveal the great potential of combining epigenetic modulation and adoptive transfer of genetically engineered T lymphocytes targeting NY-ESO-1 or other CTAs for the development of a specific immunotherapy for CRC.

Another approach could also include the introduction of sequences capable of disrupting long α-helical stretches in the regions outside the potential epitopes (Robins & Rees, 2001). Such approaches have the potential to improve the treatment response in patients with non-reactive type CRC. However, several challenges remain to be overcome, including the insufficient antitumour responses due to immunosuppression driven by T lymphocytes known as regulatory T cell (Tregs).

1.10 Infiltrating T lymphocytes

During the last decade, the search to understand the causes underlying presentation CRC has focused on Tregs. They have been shown to play a major role in cancer immunoevasion by directly inhibiting or even eradicating both CTLs and T helper lymphocytes (Weinberg, 2007). Tregs suppress autoreactive T cells without killing them through incompletely understood, contact-dependent mechanisms (Curiel, 2007). In healthy individuals they represent 5% to 10% of the

population of $CD4^+$ lymphocytes. However, in cancer patients, Tregs may increase up to 30% and are predominantly found among the tumour infiltrating lymphocytes present (Weinberg, 2007). Therefore, the failure of the T and B cells to recognize and eradicate immunogenic cancer cells could be accounted to the ability of certain tumours to secrete chemokine CCL22 which recruits Tregs and immobilizes the function of the anti-cancer immune response. Indeed patients with ulcerative colitis and irritable bowel syndrome have been shown to have higher numbers of infiltrating T cells than healthy controls (Holmen *et al*, 2006) which may play a role in controlling cancer-driven inflammation. Tregs in CRC have been shown to exhibit both pro- and anti-tumour activities governed by the level of inflammatory stimuli received and dependent of the phase of tumourigenesis (early or late) (Mougiakakos, 2011). Following treatment in a phase II clinical trial for CRC patients, the level of circulated Tregs was reported to have almost reached normal levels accompanied by 70% increase in CTLs responses against CEA epitopes (Correale *et al*, 2005). Therefore, it still remains to be determined whether presence of Tregs in CRC plays a pro- or anti-tumour role, how this correlation changes with the stage of the disease and the clinical significance of these changes.

In addition, Tregs appeared to be highly specific for a distinct set of TAAs in CRC patients, suggesting that Tregs exert T cell suppression in an antigen-selective manner (Bonertz *et al*, 2009). Several key hallmarks of Tregs in CRC have been identified, highlighting their complex role in the progression of the disease, the survival prognosis and their potential as therapeutic targets. Overall, it was established that CRC patients develop multivalent and individual T cell responses against a broad variety of different CRC-associated TAAs. Therefore, selecting a panel of antigens according to pre-existing T cell responses as an intermediary could improve the efficacy of future immunotherapies for CRC and should be further investigated. Better understanding of the role and

behaviour of Tregs within CRC would improve tumour profiling on an individual basis and could aid the choice of the most adequate therapy for each individual case. It could also benefit the development of cancer vaccines and other immune-based therapies targeting particular CTAs in non-reactive type CRC.

Tumour-infiltrating $CD45RO^+$-cell density is a prognostic biomarker associated with longer survival in CRC patients, independent of clinical, pathological, and molecular features (Nosho *et al*, 2010). Similar findings have also been reported from another study, linking the density of $CD45RO^+$ memory T cells in the different regions of CRC with dissemination to lymphovascular and perineural structures and to regional lymph nodes in patients – low densities were associated with a very poor prognosis (Pages *et al*, 2005). However, not only was the type and density of the infiltrating immune cells within human CRC predictive of clinical outcome but also their location within the tumour i.e. whether they are located in the centre of the tumour or the invasive margin. A strong *in situ* immune reaction in both regions was shown to correlate with a favourable prognosis regardless of the stage (I, II and III CRC) (Galon *et al*, 2006). Conversely, a weak *in situ* immune reaction predicted a poor clinical outcome even in patients with stage I CRC. This data was further supported by Pagès *et al* 2005 who examined the relationship between the presence of $CD8^+$ and $CD45RO^+$ in two regions of the tumour in patients with early stage CRC with regards to tumour recurrence and overall patient survival. High densities of both $CD8^+$ and $CD45RO^+$ correlated significantly with lower rates of CRC recurrence and increased overall survival when compared to patients with low densities of both immune cell types within the primary tumours. In contrast, extramural vascular invasion and high $FOXP3^+$ cell density in lymphoid follicles were independent factors for worse survival, whereas a high frequency of lymphoid follicles in histologically normal colonic mucosa was associated with better survival (Salama *et al*, 2012). Therefore, the collective

analysis of the type, density and the location of immune cells within the CRC could be used to predict patient survival and to identify the high-risk patients who would benefit most from adjuvant therapy.

1.11 Intrinsically disordered proteins (IDPs)

Following a bioinformatics approach implementing the application of two algorithms (FoldIndex and RONN) predicting the level of disorder within a sequence, the experimental outcome revealed that more than 90% of the examined 228 CTAs were IDPs (Rajagopalan *et al*, 2011). The latter are proteins that lack the typical hydrophobic cores and therefore do not appear as having rigid 3D structures (along their entire length or in localized regions) under physiological conditions and instead, exist as dynamic ensembles (Rajagopalan *et al*, 2011; Marsh *et al*, 2010). However, IDPs can evade being detected as "misfolded" and degraded by the cell's surveillance system through their ability to undergo "disorder-to-order" transitions upon binding to biological targets – a paradox known as the "order/disorder paradox" (Kulkarni *et al*, 2011). This is achieved as a segment of an IDP initially binds with the target, followed then by coalescing of the other protein segments facilitating the IDPs' folding (Zhou *et al*, 2012). However, recent studies have challenged this general view by revealing a phenomenon – uncoupled binding and folding of IDPs (Sigalov *et al*, 2011). The complexity of the binding mechanisms of IDPs has been investigated by others (Zhou, 2012; Brown *et al*, 2011) and a summary of possible pathways have been outlined in Figure 1.2.

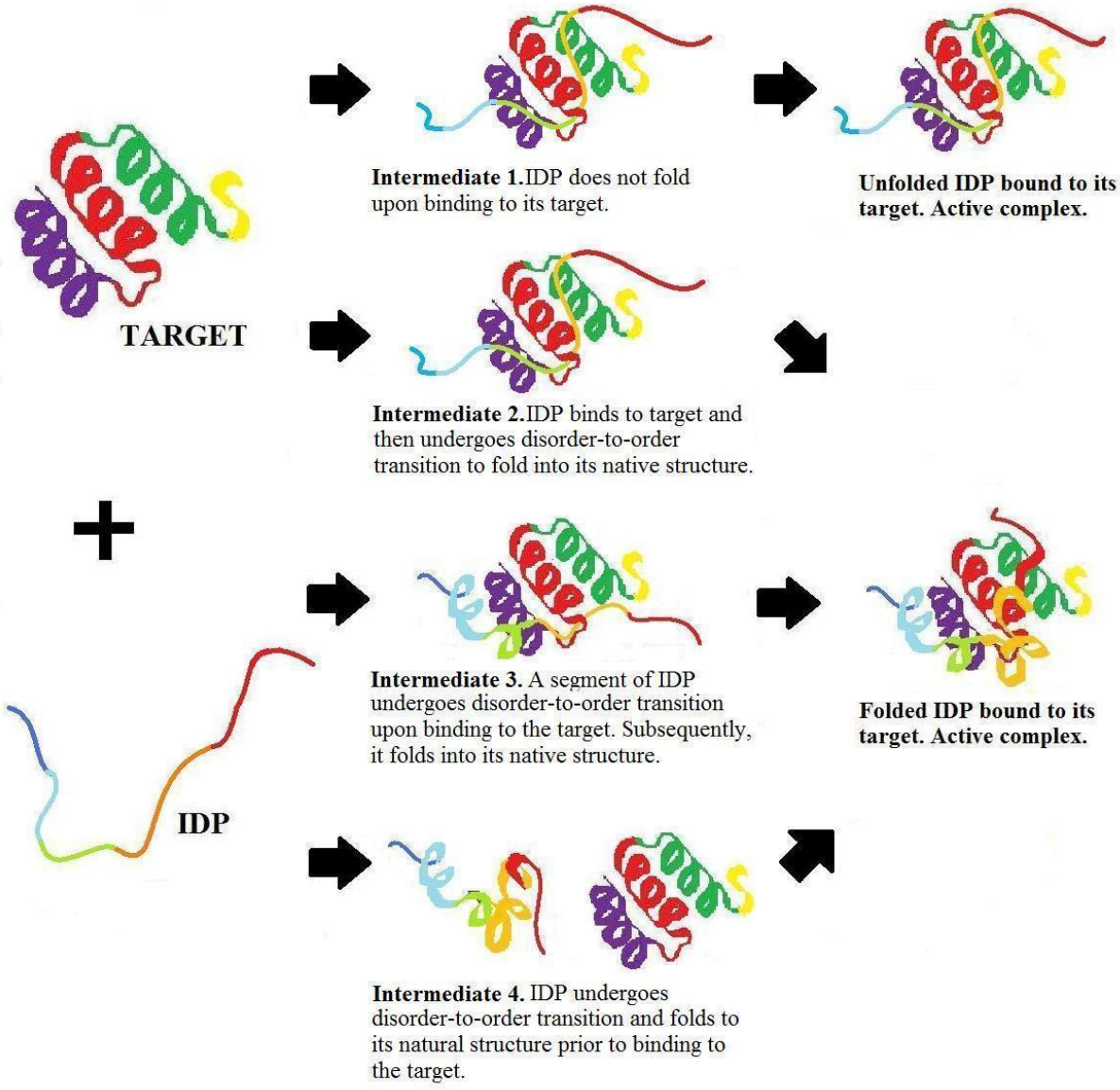


Figure 1.2 Diagrammatic representation of the different binding mechanisms and the disorder-to-order transition that the IDPs undergo prior to and upon binding to their targets (based on Sigalov, 2011). Some IDPs (particularly immune signalling-related IDPs) do not fold upon binding to their targets (for example Intermediate 1). Other IDPs undergo partial or complete folding upon binding (Intermediate 2 and Intermediate 3) or fold prior to binding to their targets (Intermediate 4). The images shown are representative of the disorder-to-order transition that a hypothetical IDP would undergo.

The properties associated with IDPs give an interesting angle of perception towards the expression, behaviour and function of CTAs identified as IDPs. The lack of rigid 3D structures is believed to be responsible for the exposure of primary contact sites which enables the faster, more effective and promiscuous binding at high concentrations to target molecules (Marsh et al, 2010). Together with the fact that intrinsic disorder has been identified as a determinant of genes that are harmful when overexpressed (Zhou, 2012), this could be accounted to the correlation between CTAs overexpression and disease prognosis. This is further supported by the fact that CTAs appear to occupy 'hub' positions (highly connected protein nodes) within the complex protein-protein interaction (PPI) network (Rajagopalan et al, 2011; Midic et al, 2009). What was interesting in these findings was the fact that these networks are dynamic and grow incrementally by establishing new nodes. However, a desirable protein for recruitment to a hub position is a protein that is likely to participate in a large number of promiscuous interactions when overexpressed such as a CTA that is an IDP (Rajagopalan et al, 2011). Eventually, the PPI network becomes dominated by such hubs leading to the overexpression of CTAs and to the creation of nodes with novel functions. This is accountable for the poorer disease prognosis in later CRC stages and the phenotypic presentation of non-reactive CRC disease. This idea is further supported by the fact that high abundance of IDPs is believed to result in undesirable interactions and potentially harmful effects of such interactions (Zhou, 2012). Perhaps the failure to respond to treatment in non-reactive type CRC could be due to targeting common nodes in a protein network rather than CTAs that occupy hub positions. Therefore, the search for such antigens could potentially take a turn towards more thorough investigation of the structure of the PPI networks established in cases of reactive disease.

1.12 Study Aims

In this study we aimed to identify the differences in the immune response demonstrated by CRC patients who respond well to conventional treatment and those who do not. To do this we used SEREX to determine which antigens were recognised by patients with reactive disease and to compare them with the antigens recognised by patients with non-reactive disease (poor responders. We hoped to identify new biomarkers of disease and potential targets for immunotherapy.

Please note: This Introduction was published as part of a review by Boncheva, V., Bonney, S.A., Brooks, S.E., Tangney, M., O'Sullivan, G., Mirnezami, A. & Guinn, B.A. (2013) New targets for the immunotherapy of colon cancer – does reactive disease hold the answer? *Cancer Gene Therapy*, **20**, 157-168 (see Appendix I). This Introduction contains work entirely written or images drawn by Viktoriya Boncheva, the author of this MPhil thesis, with supervisory guidance from Dr Barbara Guinn.

CHAPTER 2 Materials and Methods

2.1 First round of primary immunoscreening of a testes cDNA library (SEREX)

2.1.1 Primary immunoscreening

A testis cDNA expression library (constructed on 18/07/2011) was prepared by Dr Barbara Guinn while working at the Southampton University Hospitals Trust. Primary immunoscreening of that library with allogeneic colon cancer serum (Serum ID: CC010/1:10 dilution in 1xTris-buffered saline (1xTBS/0.01% NaAzide) diluted down to 1:100 in 1xTBS) was carried out as previously described (Sahin *et al*, 1995). Briefly, on Day 1, a single *E. coli* XL1-Blue MRF' colony isolated from LB Tetracycline agar plate was grown in 5ml of LB broth (supplemented with 0.2% Maltose and 10mM autoclaved $MgSO_4$) for 4 to 5 hours at 37°C and 200rpm until approximately 0.5-0.7 optical density at 600nm wavelength (OD_{600}) was reached. Subsequently, 600µl of the bacterial culture was inoculated with an aliquot of recombinant phages (testis cDNA expression libraries L1 or L2) and incubated at 37°C in a water bath for 15 minutes to allow infection to occur. Expression of recombinant proteins was induced with the addition of 20µl of 1M isopropyl-β-D-galactoside (IPTG; Melford Laboratories, UK) and the *E. coli* XL1-Blue MRF' cells were mixed with 7ml-8ml of NZY top agar (100ml of NZY broth (5g NaCl, 2g, MgSO4, 5g yeast extract, 10g NZ Amine A in 1L dH_2O (Sigma-Aldrich U.K.) at pH 7.5, autoclaved) boiled with 0.7g bacteriological agar (to produce 0.7% agar) (Sigma-Aldrich) with 2ml of filtered 10% Maltose and 5ml of filtered dH_2O)

and plated at a density of 2000-4500 pfu onto 140mm NZY agar plates (5g NaCl, 2.5g, MgSO4, 5g yeast extract, 10g NZ Amine A in 1L dH_2O at pH 7.5, autoclaved) (**Figure 2.1**).

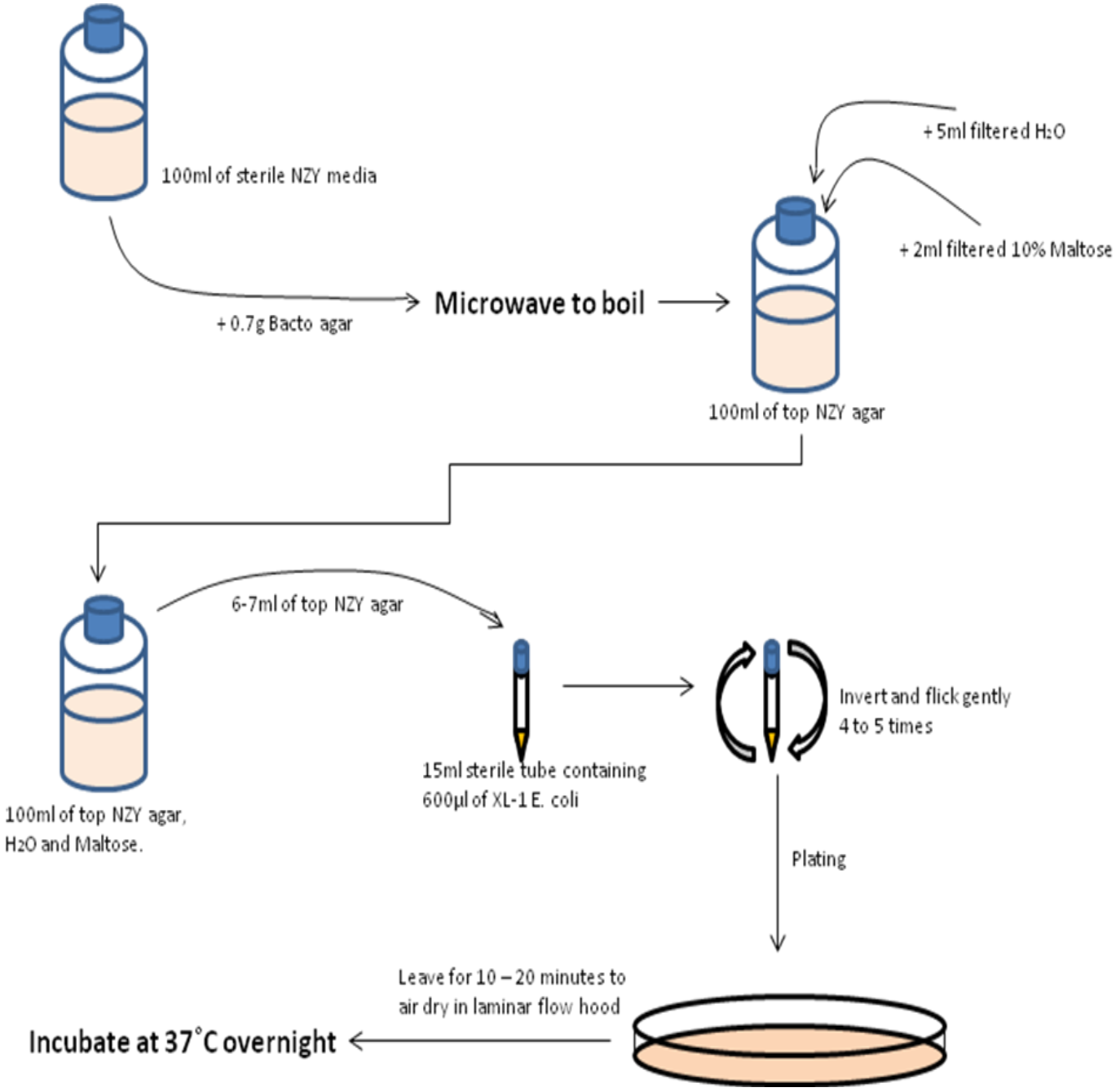


Figure 2.1 Dagrammatic representation of how NZY top agar was prepared and phage supernatant added to it as described in section 2.1.1.

Plates were incubated at 37°C overnight. On the following day, BioTrace™ NT nitrocellulose transfer membranes (0.2µm pore size; PALL Life Sciences, UK) were placed on the agar for 3 hours at 37°C to blot the recombinant proteins.

Following the incubation period (Day 2), membranes were pricked with a sterile needle (**Figure 2.2**) to allow plaque localization at later stages, removed and washed in a tray, containing TBS-Tween 20 (TBS-T) (10x TBS pH 7.4 purchased from VWR and 0.05%/0.5ml Tween-20 purchased form Sigma-Aldrich – per 1L). Any residual bacterial cell lysate or unbound phage were removed by gently sweeping across the membrane. The membranes were replaced in 150mm petridishes, containing TBS-T and placed on a rotating platform for 10-15 minutes. The TBS-T was then replaced with TBS (same as TBS-T without Tween-20) and plates were placed back on the rotating platform for another five minutes.

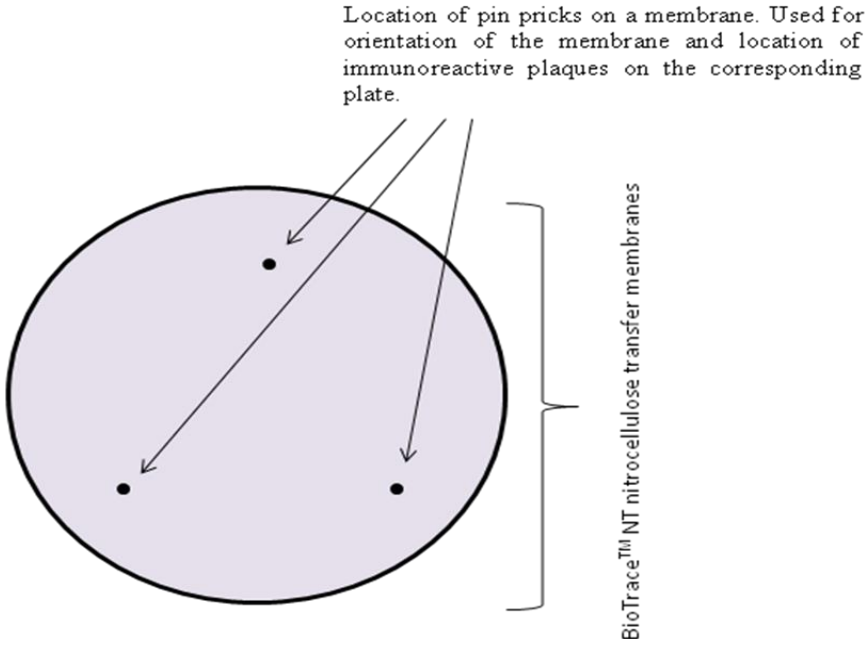


Figure 2.2 Location of needle pricks on a membrane. To enable the accurate location of the membranes with regards to positive plaques pin pricks were placed through the membrane into the NZY agar below to allow orientation. A compass was then used to locate positive plaques on the immunoscreened membrane on the NZY plate later.

Membranes were then blocked in 5% (w/v) low fat milk in TBS for 1 hour followed by four times TBS-T and one time TBS washings (each wash carried out for five minutes on a rotating platform at room temperature (RT)). The membranes were incubated overnight in 20ml of the cleaned CC010 serum (1:100 dilution).

On the following day (Day 3), the membranes were washed (as described above) and incubated with alkaline phosphatase conjugated rabbit antihuman IgG, Fc_{γ}, fragment specific antibody (Pierce, UK; Prod. No. 31318) diluted 1:5000 in 0.5% (w/v) low fat milk in TBS (10ml to each plate) for three hours, RT. Membranes were then washed (as before) and reactive plaques were visualized in 10ml of alkaline phosphatase (AP) buffer (12.1g Trizma base, 5.844g NaCl, 1.0165g $MgCl_2.6H_2O$; Sigma-Aldrich) at pH 9.5 containing 33µl of 50mg/ml 5-bromo-4-chloro-3`-indolylphosphate sodium salt (BCIP: 50mg per 1ml of dH_2O) (Sigma-Aldrich) and 330µl of 10mg/ml nitroblue tetrazolium (NBT: 10mg per 1ml of dH_2O) (Sigma-Aldrich). Immunoreactive plaques were excised off of the agar with the aid of sterile scalpels alongside several non-reactive plaques (used as negative controls in secondary screening) and eluted (in 1.5ml sterile eppendorf tubes) in 500µl autoclaved SM buffer (5.8g NaCl, 2g $MgSO_4.7H_2O$, 50ml 1M Tris.HCl at pH 7.5, 5ml of 2% (w/v) gelatin (stored at 4°C) in 1L of dH_2O) for one hour at RT, 200rpm. Subsequently, 10µl of Chloroform (Sigma-Aldrich) were added to each 1.5ml eppendorf tube. Tubes were then vortexed, centrifuged at 10,000rpm for one minute and stored at 4°C for secondary immunoscreening. Plaques on each plate were counted by dividing the plate into four equivalent quadrants, noting the number of plaques present in one of the quadrants and multiplying by four to give an average number of plaques for the plate.

2.1.2 Secondary immunoscreening

Positive phages were rescreened by infecting 200µl of *E. coli* XL1-Blue MRF' cells at 0.5-0.7 OD_{600} with 5µl of phage solution and plating with 2ml to 3ml of top NZY agar and 8µl of 1M IPTG onto NZY agar plates. Subsequently, steps were followed as in primary screening. Any positive staining plaques were to be isolated to monoclonality with the aid of a glass Pasteur pipette, eluted in 500µl autoclaved SM buffer and stored at 4°C for sequencing and tertiary screening.

2.1.3 Preparation of reagents used for Primary and Secondary immunoscreening

- LB broth. The LB broth (Lennox L broth) was prepared by adding 1 LB broth tablet (10g/L Tryptone, 5g/L Yeast Extract, 5g/L NaCl and 2.2g/L inert binding agents; Sigma-Aldrich) per 50ml of deionized water (dH_2O) (20 tablets for 1L of dH_2O) and autoclaving at 121°C for 20 minutes to produce sterile LB broth with pH of 7.5.

- LB tetracycline plates. The LB tetracycline plates were prepared by autoclaving 100ml of LB broth (preparation as described above) with 1.5g Bacteriological agar (70% agarose and 30% agaropectin; Sigma-Aldrich) to produce 15% agar. Subsequently, the LB agar was left to cool down to around 50°C and following the addition of 125µl of Tetracycline (12.5mg/ml diluted in 70% EtOH) approximately 20ml of the agar were poured in a 90mm petridish (avoiding the formation of bubbles) and dried for 30 minutes in a laminar flow cabinet. The dried agar plates were wrapped up in a plastic bag and stored at 4°C upside down for future use.

- Inoculation of LB tetracycline plates. Two LB tetracycline plates were inoculated with *E. coli* XL1-Blue MRF' cells (**Figure 2.3**) every two weeks to maintain fresh bacterial stock. Older colonies would take longer than 5 hours to reach 0.5-0.7 OD_{600} in 5ml of LB broth.

- 1M IPTG. A stock of 1M IPTG was prepared by vortexing 238mg of IPTG powder with 1ml of dH_2O until homogenous solution formed. The stock was kept at -20°C for further use. It is recommended to keep small aliquots of IPTG (around 200µl) as it cannot be freeze thawed too often and also away from direct light (tube could be wrapped up in thin foil) as IPTG is light sensitive.

- 10% Maltose. A 50ml stock solution of 10% Maltose was prepared by vortexing 5.1g of 98% Maltose (powder; Sigma-Aldrich) with 50ml of dH_2O until clear homogeneous appearance was reached. The stock solution was then filtered through a 0.2µm syringe filter (Fisher) in a laminar flow cabinet into a sterile 50ml Falcon tube (Fisher) and were filtered again prior each use.

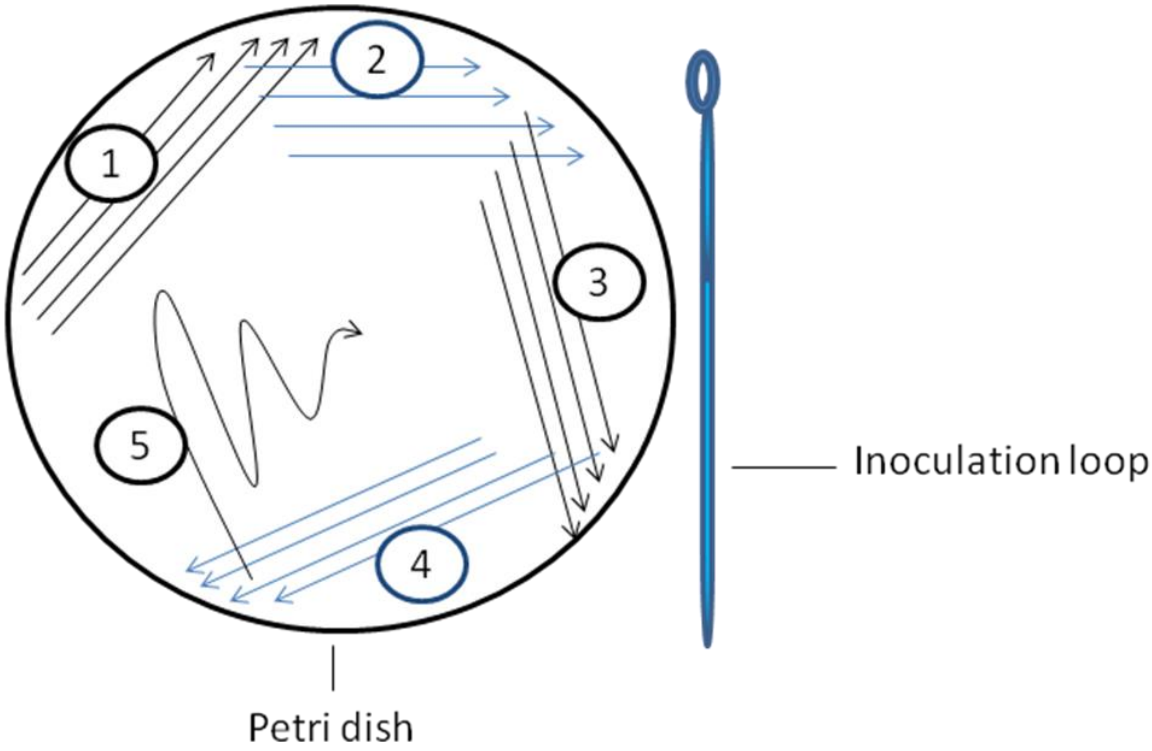


Figure 2.3 Diagrammatic representation of the method employed when spreading a single *E. coli* XL1-Blue MRF' colony onto a LB Tetracycline plate. This technique enabled the isolation of individual colonies following an overnight incubation at 37°C. Each of the five steps consisted of four individual streaks (the first of each four crosses with the fourth streak of the previous four) with an inoculation loop on the LB agar.

- 1M $MgSO_4.7H_2O$. A 200ml stock solution of 1M $MgSO_4.7H_2O$ was prepared by mixing 24.07g of $MgSO_4.7H_2O$ with 200ml of dH_2O and autoclaving at 121°C for twenty minutes. The solution was kept at RT and syringe filtered every time prior to its use.

2.2 Determination of serum reactivity and testis cDNA library efficiency

The results obtained during the first round of primary and secondary immunoscreening with SEREX required further investigation to establish the quality of the pooled serum (CC010) and also the efficiency of the phage cDNA library used in this investigation. Serum reactivity was determined through cross-matching the serum with animal whole blood and cDNA library efficiency was tested via blue/white screening for recombinant plaques and via PCR.

2.2.1 Blood agglutination and serum reactivity test

Erythrocyte agglutination is a phenomenon observed in cases of cross-matching of erythrocytes and serum of incompatible organisms and therefore is a well-established method used predominantly for blood typing (Landsteiner, 1900). The basic principle of the technique was applied in this investigation – we wanted to demonstrate the reactivity of the patients sera we were using for immunoscreening. Animal blood samples and a human blood sample of known type (A+)(stored at 4°C) were mixed with the cleaned CC010 serum (1:10 dilution)(**Figure 2.4)**. Samples were observed for signs of agglutination under a light microscope at 1000x magnification and compared to control samples containing only the animal or known blood type sample or the same mixed with 1xPBS (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4 and 0.24g KH_2PO_4, pH 7.4). Following the addition of serum/1xPBS to the corresponding blood samples, the glass slides were kept for 2-3 minutes at RT

prior to observation under light microscope (see **Figure 3.1** in Results). If blood samples appeared dry the agglutination test was repeated with a shortened incubation time at RT.

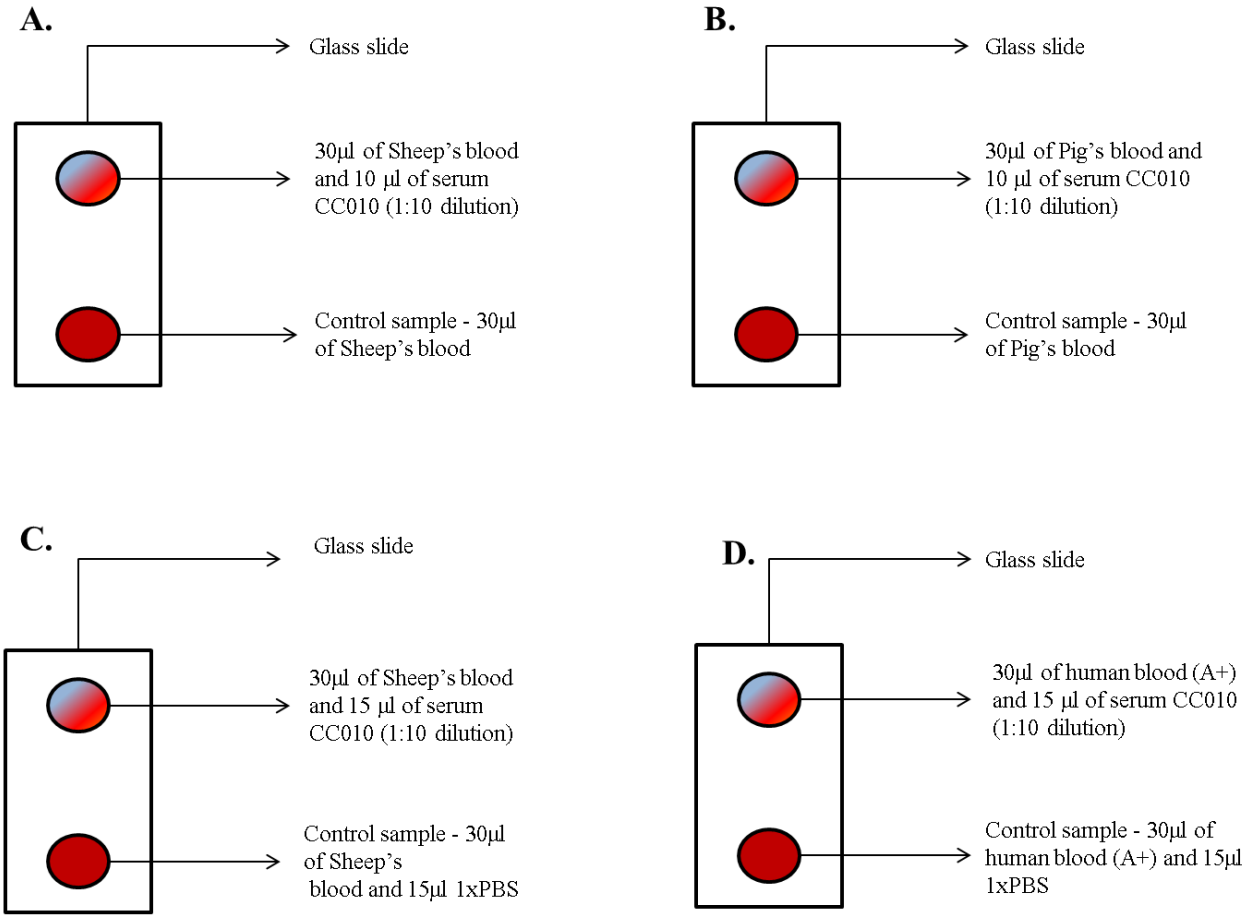


Figure 2.4 **Serum reactivity test.** Animal blood or human blood of a known group were mixed with pre-cleared Dukes' B serum used for immunoscreening**.** Agglutination indicated the presence of active antibodies in the Dukes' B sera.

All surfaces and microscope lenses were disinfected with 70% EtOH and slides discarded correctly at the end of each agglutination test. Extreme caution was carried out while handling the blood

samples and glass slides and personal protective equipment (PPE) – gloves, goggles and laboratory coat were worn at all times. Blood samples were discarded in the infectious waste bin alongside with disposable PPE and other materials.

2.2.2 Blue/white screening for recombinant plaques

Blue/white screening with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma-Aldrich) was carried out to establish whether the cDNA phage library used in the first round of SEREX primary screening has been packaged efficiently. Following infection of XL1-Blue MRF' bacterial cells with cDNA phages in the presence of X-gal is expected to result in the formation of colourless plaques where a recombinant phage was present and in the formation of blue plaques where the infectious phage did not contain a cDNA insert. To set up the experiment a single colony of XL1-Blue MRF' was grown in 5ml of LB media as previously described until OD_{600} of 0.547 was reached. Four 150mm petridishes were prepared as previously described in Day 1 of SEREX primary screening (each plate to screen a different cDNA library so in total four libraries were screened), with the addition of X-gal (dissolved in DMSO (dimethylsulfoxide; Sigma-Aldrich)) at two different concentrations as detailed in **Table 2.1**.

Plates were removed from the incubator on the following day and the plaques were observed (**Figure 3.2**). This experiment was repeated on another occasion and the outcome was affirmative of the original findings.

Table 2.1 X-gal screen for recombinant phages

In order of addition:	Plate 1	Plate 2	Plate 3	Plate 4
XL1 - blue at OD600 of 0.547	600µl	600µl	600µl	600µl
cDNA library (known to give around 3500pfu per plate)	10µl of L1	7µl of L2	1µl of T27	1µl of T28
Incubation at 37˚C water bath	15 minutes incubation			
1M IPTG	20µl	20µl	20µl	20µl
50µl of X-gal at concentration of:	50mg/ml	220mg/ml	50mg/ml	220mg/ml
Plating	mixed with 6ml of NZY top agar and plated			
Incubation at 37˚C overnight	Around 12 hours			

2.2.3 PCR to determine presence of cDNA insert in libraries L1, L2, T27 and T28

The results from the blue/white selection were further confirmed through PCR by using the four cDNA phage libraries as templates and T3/T7 primer pair as described earlier (SEREX). PCR was set as follows:

In each PCR tube - 12.5µl of ReadyMix (without $MgCl_2$) (Sigma-Aldrich), 1.5µl of $MgCl_2$, 0.5µl of primer T3, 0.5µl of primer T7, 1µl of template (L1 in tube 1, L2 in tube 2, T27 in tube 3 and T28 in tube 4) and 9µl of H_2O (provided from Sigma-Aldrich) – final volume 25µl in each PCR tube. A

positive control reaction was set with the above solutes and with 0.5µl of GTK-AML-8 plasmid (with binding sites for T3 and T7) and 9.5µl of H_2O to final volume of 25µl. One negative control (containing no template) was used with 10µl of H_2O to final volume of 25µl. The so prepared samples were placed in a PCR machine at the following program: 94˚C for 2 minutes, followed by [94˚C for 30 seconds, 51˚C for 40 seconds, 72˚C for 40 seconds] x 40 cycles and 72˚C for 7 minutes. Following the completion of the PCR cycles, the samples were analysed on a 1.5% agarose gel (1.5g agarose powder (Sigma-Aldrich) in 100ml of 1x TAE buffer (50x TAE purchased from Fisher Scientific, Loughborough, England and diluted in distilled water) and boiled until homogeneous in a microwave; 5µl of EtBr (Sigma Aldrich, UK) was added per 100ml of 1.5% agarose solution after it has been cooled down to around 50°C. The agarose solution was mixed thoroughly, avoiding the formation of bubbles and poured gently on a gel tray to allow solidifying). Each sample was mixed 1:6 with a 6x loading dye (3ml glycerol (30%), 25mg bromophenol blue (0.25%) and dH_2O to 10mL) was prepared with reagents from Sigma-Aldrich) and loaded into a corresponding well of the gel. The gel tank was switched on and alolowed to run for 45 minutes at 100V. The gel was observed under UV light (Image 3.3).

2.3 Construction of a new testis cDNA library

2.3.1 Ligation of cDNA inserts in ZAP Express Vectors and packaging

Several attempts were made to construct a new cDNA phage library before the reaction was successfully optimized to give a positive result. The testes cDNA inserts (already processed according to ZAP Express cDNA Synthesis Kit Protocol from Stratagene, UK) were kindly provided by Dr Barbara Guinn (University of Bedfordshire, UK). They were prepareded in

September 2009 at the University of Southampton and stored at -80°C until required. Following the optimisation of the reactions, the first step of the construction of new cDNA expression library were the ligation of the cDNA inserts into the ZAP Express Vector according to the protocol provided by Stratagene UK usingl reagents from the ZAP Express cDNA Synthesis Kit.

The reaction was performed as follows: 4µl cDNA (100ng DNA) (from 09/09), 0.5µl ligase buffer, 0.5µl 10mM rATP, 1µl ZAP Express Vector, 0.5µl T4 DNA ligase (4 U/µl) were added to a sterile 1.5ml Eppendorf tube. The mixture was incubated at room temperature for two hours.

Following incubation, 5 µl of ligation were packaged into a Giagapack III Gold Packaging extract according to the protocol provided by Stratagene UK. The packaging extract was thawed (by holding it between the fingers) from -80°C before the ligated cDNA was added to it. The mixture was stirred very carefully with the aid of a sterile pipette tip and centrifuged at 10000rpm for 3-4 seconds. The mixture was then incubated at room temperature for ninety minutes. Following the incubation, 250µl of autoclaved SM buffer and 10µl of Chloroform were added to the ligation mixture. The tube was vortexed at low speed, centrifuged at 10000rpm for one minute and then stored at 4°C.

2.3.2 Plating and titering the new library

Following the instructions on Stratagene protocol, *E. coli* XL1 Blue were grown to OD_{600} of 0.5-0.8 and infected with the newly constructed testes library cDNA containing phagemid. Four 150mm NZY agar plates were prepared as described using 1µl, 1.5µl, 2.5µl and 3.5µl of the cDNA library respectively per 600µl of *E. coli* culture. 10µl of 1M IPTG and 40µl of X-gal (250mg/ml in DMF) were added along with 7-8ml of NZY top agar to the bacterial culture prior to plating. The four plates were then incubated at 37˚C overnight to allow the formation of plaques. Following

incubation, the white and the blue plaques were counted separately and together to give a total number of pfu per 1µl (**Table 3.1**).

2.3.3 Amplifying the ZAP Express library

Amplification of the primary library was carried out on the following morning as the primary cDNA libraries tend to be unstable and lose efficiently. The manipulations were carried out according to the protocol provided by Stratagene. *E. coli* was grown to OD_{600} of 0.5-0.8 and four 600µl aliquots of the bacterial culture were placed in four 15ml sterile Falcon tubes. The first three were infected with 35µl of the primary cDNA library and the fourth one – with 45µl. Each mixture was supplemented with 20µl of 1M IPTG and 6-7ml of NZY top agar then poured onto 150mm NZY plate. The plates were incubated overnight at 37˚C to allow the plaques to form (approximately 2mm in diameter) and the agar was overlayed with 6ml of sterile SM buffer. The plates were placed on a rotating platform at 4˚C for 12 hours. The SM buffer was collected from the plates in a sterile environment and was pooled together. Chloroform was added to the suspension at 5% of total volume and the amplified library was left at room temperature for 15 minutes. The suspension was centrifuged for 3 minutes at 5000rpm and the clear supernatant was replaced into two new, sterile 15ml Falcon tubes. Chloroform at 0.3% of final volume was added, tubes were briefly vortexed and centrifuged and then stored at 4˚C.

This procedure was repeated with the remaining 140µl of primary cDNA library (following ligation in ZAP Express vector and packaging). Three plates were prepared as described above with 40µl of primary cDNA library used for each one. The plates were overlayed with 8ml of sterile SM buffer and incubated. The buffer was collected after overnight incubation on a rotating platform and processed as described above.

The amplified library was then screened with X-gal (blue/white screening as previously described) to check efficiency and recombinancy. Eight NZY plates were prepared, using two random library aliquots for the infection of XL1 Blue bacterial cells (1µl of 1:10 dilution, 5µl of 1:10 dilution, 1µl of 1:50 dilution and 1µl of 1:100 dilution of each aliquot). Following the incubation time, the total number of plaques (sum of white and blue plaques) was determined (**Table 3.2**).

The amplified library was pooled together and aliquoted into 1ml sterile tubes - 300µl of cDNA library in each. DMSO (12.5% v/v) and Chloroform (7.5% v/v) were added to each aliquot and the samples were stored at -80˚C for further use.

2.3.4 Confirmation of cDNA library efficiency

A PCR reaction with T3/T7 primer pair was carried out (conditions as described above) to verify the recombinancy and efficiency of the newly constructed testis cDNA library. As non-recombinant phages are present in the main stock, several aliquots of the cDNA library were used for the preparation of four NZY plates (as described in Day 1 of SEREX protocol) in the presence of X-gal to differentiate recombinant from non-recombinant plaques. Plates were incubated and on the following day twenty two recombinant plaques were selected at random and used as templates in a PCR. The aim was to demonstrate that the newly constructed library contains cDNA inserts and that they are of various sizes (**Figure 3.5**).

2.4 Second round of primary immunoscreening with SEREX – optimised protocol

Primary immunoscreening of testis cDNA phage library with SEREX is a process which takes three days for completion. The manipulations carried out at each day were carried out as described in the

first round of primary immunoscreening with minor changes accumulated in the process of optimising the method.

Day 1

A single *E. coli* XL1-Blue MRF' colony should be isolated from LB Tetracycline agar plate (prepared as described earlier) and grown for 4 to 5 hours in 5ml-7ml of LB broth in 50ml Falcon tubes (supplemented with 0.2% Maltose and 10mM autoclaved $MgSO_4$) at 37°C and 200rpm. The tubes should be incubated until the culture reaches approximately 0.5-0.7 optical density at 600nm wavelength (OD_{600}). This step should be carried out at around 10am in the morning to allow sufficient time for the bacterial culture to reach the desired density (the amount of LB broth should be considered as larger volumes would require longer incubation times). The cultures should be placed at 4°C for at least one hour prior to any further manipulations in relation to the SEREX protocol.

The final step for Day 1 involves the infection of *E. coli* XL1-Blue (grown to the desired OD – preferably 0.6 at 600nm) with the appropriate volume of recombinant cDNA expression library to give an efficiency of around 4500 plaque forming units (pfu) per 150mm Petri dish. This process should take between thirty to fifty minutes (depending on the number of plates) if preparation and planning is carried out beforehand. The following steps were performed. On Day 1we remove an appropriate number of NZY plates (up to two weeks old) from the laboratory fridge and placed them at 37°C incubator. Plates were placed with their lids uncovering approximately 50% of the agar to allow any excess fluid to evaporate.

A number of aliquots of bacterial culture were infected with the newly constructed pooled cDNA library. 5µl of 1:10 dilution was established to produce around 4000-4500 plaques per 150mm plate. The *E.coli* and phage cultures placed in a water bath set at 37˚C for 15 minutes.

The appropriate amount of IPTG (20µl of 1M IPTG) was added to each tube of E.coli and phage library prior to pouring in the 55^{o}C NZY top agar.Tilt the 15ml tube containing bacteria, phage and IPTG had warm NZY top agar (to final volume of 6ml-7ml) added to it. The lid was closed, the tube inverted, to mix the contents evenly. The bacteria, phage and IPTG were poured onto a large NZY plate and ensure even spread by rotating and moving back-forth and left-right the plate and then left to solidify. We then closed the lids on the petri dishes and place them in an incubator (upside-down) at 37˚C overnight. Best results were achieved if incubation was carried out for 12 hours rather than the 16hrs published previously (Liggins *et al*, 2005). Plaques were visible the next morning around 2mm-3mm in diameter.

Day 2 and Day 3

The manipulations carried out on Day 2 and Day 3 of primary immunoscreening with SEREX as described in Section 2.1.1. Three allogeneic colon cancer sera were used as follows:

1. Serum ID: CC014/1:10 dilution in 1xTBS/0.01% NaAzide (pre-cleared as described in Liggins *et al*, 2005 on 10.06.2010 by Dr Barbara Guinn and stored at -80˚C) was diluted down to 1:200 in 1xTBS and NaAzide was added to it at concentration of 0.01% of the total volume. The serum was diluted on the 6.02.2013 and used for primary and secondary immunoscreening.

2. Serum ID: CC005/1:10 dilution in 1xTBS/0.01% NaAzide (pre-cleared by Dr Barbara Guinn and stored at -80˚C) was diluted down to 1:200 in 1xTBS and NaAzide was added to it at

concentration of 0.01% of the total volume. The serum was diluted on the 19.02.2013 and used for primary and secondary immunoscreening.

3. Serum ID: CC010/1:10 dilution in 1xTBS/0.01% NaAzide (pre-cleared by Dr Barbara Guinn and stored at -80˚C) was diluted down to 1:200 in 1xTBS and NaAzide was added to it at concentration of 0.01% of the total volume. The serum was diluted on the 26.04.2013 and used for primary and secondary immunoscreening.

The overnight incubation with the appropriate serum took place between 5:30pm and 11:30am.

The results of second round of primary immunoscreening with SEREX as presented in **Table 4.1** (**Section 4.1**).

2.5 Secondary immunoscreening of testis cDNA library with SEREX

Plaques, containing immunoreactive cDNA inserts were isolated to monoclonality with the aid of a glass Pasteur pipette. The end of the 180mm glass pasteur pipette was allowed to just surround the plaque (**Figure 2.5**) and then pressed firmly down through the NZY agar. The pipette was then carefully lifted and the collected plaque was eluted in 250µl of sterile SM buffer (preparation as previously described) and stored at 4°C. A new pipette was used for the isolation of each plaque.

During the first few secondary immunoscreening reactions with SEREX, three positive plaques were isolated from each membrane (resembling copies of the original positive plaque isolated through primary immunoscreening). However, as this investigation suggested on a later stage, not all of them would always be true positives, would be successfully eluted in the SM buffer or even

contain a cDNA insert. Therefore, it is advised that as many as possible immunoreactive plaques are isolated from each membrane (from secondary immunoscreening) and then run through a PCR (T3/T7 primers) to confirm the presence of cDNA inserts. Only the ones that produce a band (against positive and negative control PCR reactions) should be kept and processed further (sequencing, analysis, etc.).

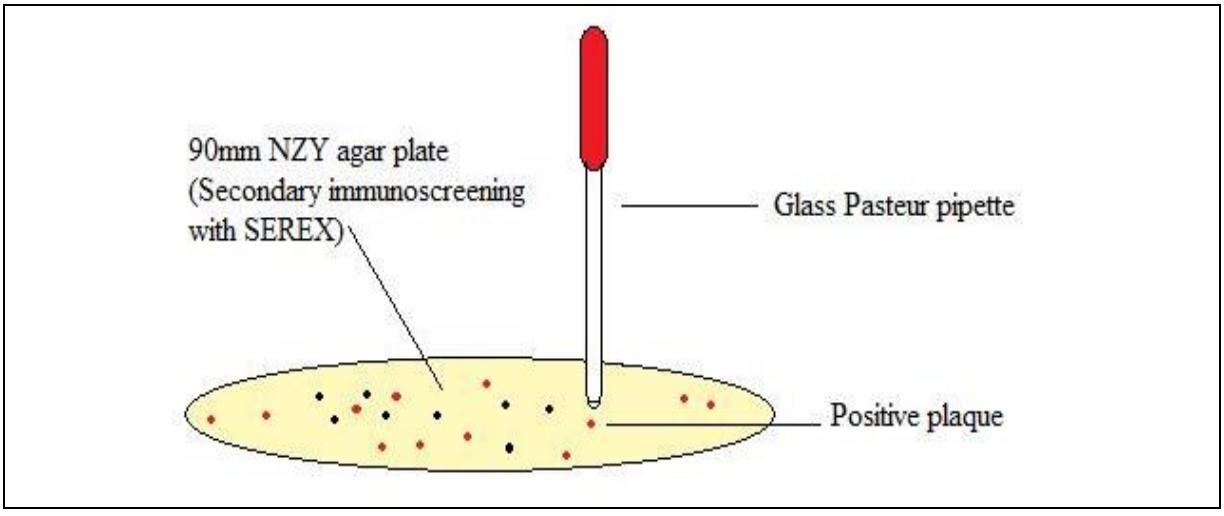


Figure 2.5 Isolation of a single positive colony following secondary screening with SEREX. A diagrammatic representation of the process of isolating positive plaques following secondary immunoscreening with SEREX. Each positive plaque was picked out from the NZY agar with a sterile glass Pasteur pipette which was discarded following its use.

2.6 Isolation of immunoreactive cDNA inserts through PCR and gel extraction

The size of cDNA inserts was determined through PCR carried out with the aid of T3 and T7 primer pair as suggested in the Stratagene ZAP Express manual. Individual PCR reactions were carried out in an MWG advanced Primus 96 PCR machine under identical conditions:

The PCR sample mix was prepared following a strict order in the addition of reagents. PCR tubes (StarLabs, U.K.) were lined up on a stand and 12.5µl of ReadyMix (Sigma-Aldrich) was added gently to the bottom of each tube avoiding touching the walls of the tube or any other surfaces. The next step involved the addition of 1µl of T3 or T7 primer (diluted to 10pMol concentration with the addition of sterile H_2O). Subsequently, the appropriate volume of H_2O was added to complete the final volume of the solution to 25µl. Each immunoreactive phage, eluted in SM buffer, served as a template for an individual PCR reaction by adding 5µl of the phage solution to the PCR sample mix. Finally, the second primer was added to each sample mix. PCR tubes were carefully closed and centrifuged for 3-5 seconds to bring the solution to the bottom. Tubes were then placed at the PCR thermo-cycler and the following PCR conditions were applied: 94°C for 2 minutes, [94°C for 30 seconds, 51°C for 40 seconds, 72°C for 40 seconds] x30 cycles, 72°C for 7 minutes and 4°C forever.

Following cDNA amplification, each 25µl product was mixed with a loading buffer (1:6) and loaded onto 1% agarose gels (prepared as previously described). Gel electrophoresis, carried out at 90V for 70 to 90 minutes, was used to determine the approximate size of the products and to separate the bands for further processing. After the gels were observed under UV light, each band, containing reactive cDNA fragment was excised of the gel with a scalpel and the product was extracted from the gel with the aid of a QIAquick Gel Extraction kit (Qiagen, UK). The method for extraction was as outlined in the QIAquick Gel Extraction kit protocol for gel cleanup with microcentrifuge. The cDNA fragments were eluted in 30µl ddH_2O and placed at 4˚C for further use.

This method was applied each time prior to sending off samples for sequencing.

2.7 Tertiary immunoscreening with SEREX

In tertiary immunoscreening with SEREX, a number of confirmed immunoreactive plaques can be immunoscreened simultaneously with the same serum or also with seras of healthy volunteers of from patients with non-reactive Duke's B. The preparation is similar to Day 1 of primary immunoscreening. XL-1 Blue E. coli is grown as described earlier to an OD_{600} of 0.5-0.7. The culture is then aliquoted at 600µl in 15ml sterile tubes and mixed with NZY top agar (without infecting it with phage cDNA library). The mixture iss then plated on 150mm NZY agar plates and left to dry in a laminar flow hood for 15-20 minutes. Following that, the top agar should be inoculated with 0.5µl of cDNA phage obtained from secondary immunoscreening with SEREX (only the individual reactive plaque) at even distances (**Figure 2.6**).

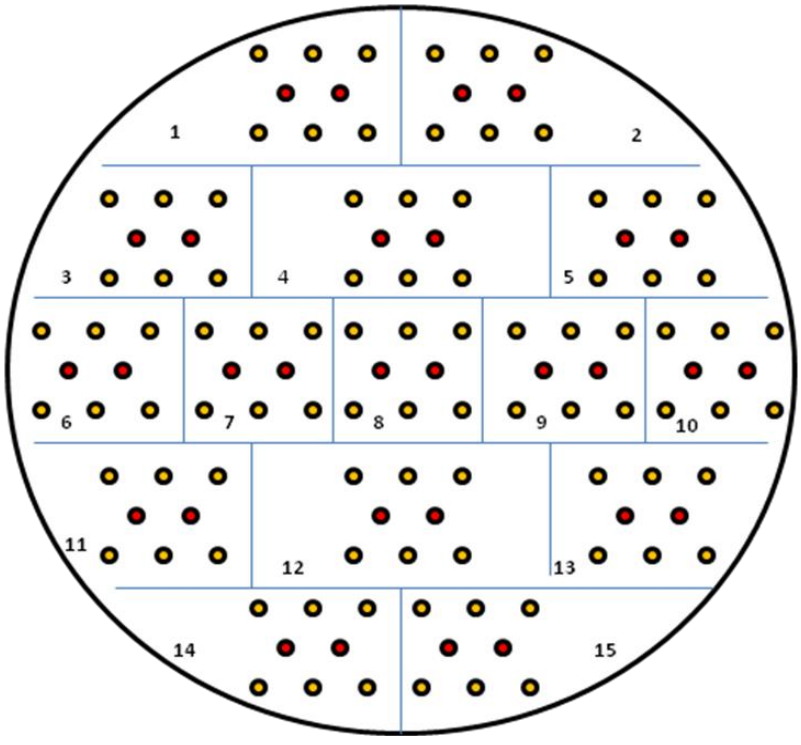


Figure 2.6 A diagrammatic representation of tertiary immunoscreening of UOB-COL-1 to UOB-COL-15 (confirmed antigens). Yellow spots represent actual cDNA samples and red spots – negative controls. Placed in a sterile environment on the top of a NZY top agar containing E.coli (no phage cDNA).

For each immunoreactive plaque (confirmed antigen UOB-COL-1 to UOB-COL-15) two rows of three 0.5µl spots should be placed. In between the two rows, two 0.5µl spots of empty phage were placed as negative controls (empty phage isolated via blue/white screening test – eluting a blue, non-recombinant plaque in SM buffer).

2.8 Sequencing and sequence analysis of identified antigens

Sequencing of the isolated PCR fragments was carried out by DNA Sequencing Facility at Cambridge University. PCR fragments were isolated as described in section 2.6 and the DNA concentration was determined with the aid of NanoDrop 2000. Samples were supplied for sequencing at concentration of 20ng per 100 base pair in 10µl of sterile H_2O. Primers for the sequencing (T3 and T7) were supplied at 10pm/µl H_2O (2µl of primer solution per reaction).

The sequence was read through BioEdit software and then placed in Ensemble Genome database to search for genetic match. Sequences were also analysed through NCBI Blast genome database for matching genes.

2.9 Cell culture

The epithelial cell line SW480 was originally derived from a 50 year old male Caucasian with Dukes' type B, colorectal adenocarcinoma (Fogh *et al*, 1977). This adherent cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) containing 4.5 g/L glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino-acids and

supplemented with 10% foetal bovine serum (FBS) and 2.5g/L amphotericin B antifungal antibiotic (Sigma-Aldrich). Cells were grown in a humidified atmosphere of 5% CO_2 at 37°C. Manipulations were carried out in a sterile environment at all times (a laminar flow tissue culture cabinet) and were washed in a sterile and RNase-free phosphate-buffered saline (PBS) (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4 dissolved in 1L of deionized water at pH 7.4 - autoclaved and stored at 4°C) during procedures and also prior to mRNA extraction.

2.9 mRNA extraction from SW480 cell line and first strand cDNA synthesis

Total mRNA from SW480 cell line pellets (10^5 cells per pellet) was extracted using RNeasy Mini Kit (Qiagen, UK). The total mRNA isolation was carried out as outlined in the protocol accompanying the RNeasy Mini Kit. Briefly, 300µl of buffer RLT was added to a loosened (by flicking the tube) SW480 cell pellet and lysis was facilitated by vortexing. The homogenized content was briefly centrifuged at 10000rpm to gather down to the bottom of the tube. Then one volume of 70% ethanol was added to the homogenized lysate and mixed carefully by pipetting. The sample was transferred to a RNeasy spin column placed in a 2ml collection tube and centrifuged for fifteen seconds at 10000rpm. The flow-through was discarded. An addition of 700µl of buffer RW1 to the RNeasy spin column was followed by centrifugation for fifteen seconds at 10000rpm and the entire flow-through was discarded. The spin column membrane was washed twice with 500µl at 10000rpm and then placed in a new 1.5ml eppendorf tube (Fisher Scientific). The total mRNA was eluted in 30µl of RNase-free water by centrifugation at 10000rpm.

2.10 Designing primers for known antigens

Firstly, the sequence of the desired gene (known antigen coding gene) was obtained from NCBI Nucleotide database [online]. The sequence was then placed in BioEdit software and the length and location of athe desired product were determined. This particular sequence was then used in YeastGenome online database to construct a pair of primers according to our preferences. The properties of each primer pair were selected manually and the database provided a choice of primer pairs. The appropriate primer pairs were selected and used for detecting expression of known antigens in SW480 cell line (Table 2.2).

2.11 Identification of antigen expression in SW480 cell line

In this investigation, SW480 cell line was screened for the expression of eight antigens: TSP50, HAGE, BCP-20, WT1, SSX2, SSX2IP, NY-ESO-1 and RAGE via PCR. Total mRNA from SW480 cell line was isolated and used for the synthesis of cDNA (as described in section 2.9). The cDNA from SW480 was used as a template in a PCR reaction (conditions to be determined with experimentation). Each primer pair was used on SW480 template (individual reactions) to detect expression of that antigen. If the antigen would have been expressed in the cell line it would have had an mRNA transcript. Therefore, there would be a presence of cDNA of that antigen in the template mixture used for PCR.

Table 2.2 PCR primer sequences for antigens investigated for expression in SW480 cell line using RT-PCR.

Antigen	Primer sequence 5'-3'	Temperature of Melting (T_m) ˚C	Estimated product length (bp)
β-Actin	F: GGCATCGTGATGGACTCCG R: GCTGGAAGGTGGACAGCGA	69.2 69.0	Not known
GAPDH	F: ACCCACTCCTCCACCTTTG R: CTCTTGTGCTCTTGCTGGG	64.0 63.8	Not known
TSP50	F: TGACGGCATGTGGCCTCAGTT R: TCAGAGGGCAGCAAGGAGGCT	72.3 71.7	401
HAGE (near 3' end)	F: CTGTACTAGTGGGGTAGAGAATTCA R: CACAGAAACGAACATTTATTAACAG	61.3 60.4	351
HAGE (near 5' end)	F: TTTGTTGGCGCGGTAATCGGT R: CATGGCACTTGTGGCAGTGGA	72.1 71.8	351
HAGE	F: CCTTTCAATGTTATCCTGAG R: TATTCTTCAGATTGACGAAG	56.2 54.4	Not known
BCP-20 (near 3' end)	F: ATGAGTCACTCGACGAGGAGC R: TTTAAAGCCACCTGGAGCCT	65.9 64.7	451
BCP-20 (middle)	F: TCATTCATCAGCAGCTTGAGC R: GAACGTGGCATTTGATGTTGA	65.6 65.8	301
WT1	F: GAGAGCGATAACCACACAAC R: GATGACCAAACTCCAGCTGG	59.8 64.7	Not known
SSX2	F: AAAATCAGAGTCAGACTGCTCCCGGTG R: GTACATGCTGACCAGGAAACAGAGTGA	73.5 70.0	Not known
SSX2IP	F: TGAATGAGCTGCTTGTGCTT R: GCTGATGCAAATTCCTGTTCT	63.7 63.1	Not known
NY-ESO-1	F: CCCCACCGCTTCCCGTG R: CTGGCCACTCGTGCTGGGA	72.6 72.4	Not known
RAGE	F: GTGTCTCCTTCGTCTCTACTA R: GGTGTGCCGATGACATCG	55.1 66.7	Not known

CHAPTER 3 Results: First round of immunoscreening with SEREX

3.1 First round immunoscreening with SEREX

The first round of primary and secondary immunoscreening with SEREX did not yield any immunoreactive plaques. Following the SEREX protocol, **259,754** plaques were screened and **73** potential positives were isolated and further examined. However, none of them was confirmed to be immunoreactive following secondary immunoscreening with SEREX. To find out the reason for the lack of positive reaction, several tests were carried out. The main concerns being the quality of the cDNA library and the reactivity of the patient serum used in these investigations:

The serum reactivity test revealed that the patient serum triggered an immune reaction that resulted in agglutination when added to a sample of sheep whole blood (**Figure 3.1**). Although, not entirely conclusive, the result was indicative that the efficiency of the cDNA library itself should be investigated.

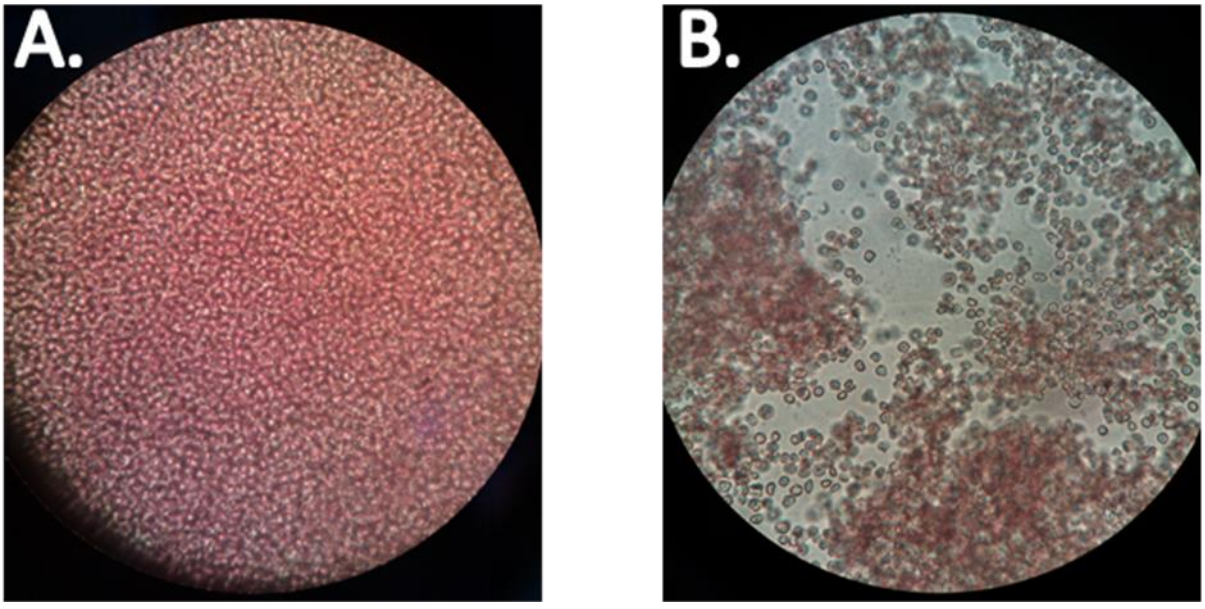


Figure 3.1 Serum reactivity test. Control sample containing 30µl of sheep blood and 15µl of normal PBS saline **(A)** and test sample containing 30µl of sheep blood and 15µl of CC010 patient serum diluted 1:100 **(B).** Although, the test is improvised, the sheep blood appeared to have reacted against the foreign sera. It appears that the serum is reactive and capable of eliciting an immune reaction **(B)** Samples observed at 1000x magnification under light microscope.

Following the serum reactivity test, the efficiency of the cDNA library was investigated. A blue/white screening was carried out to determine the presence of cDNA inserts within the phages contained in the testis cDNA library. As a result, 100% of the plaques formed within the NZY agar appeared blue (**Figure 3.2**), indicating that the phages within the currently used cDNA library were non-recombinant.

The test was repeated with the same findings.

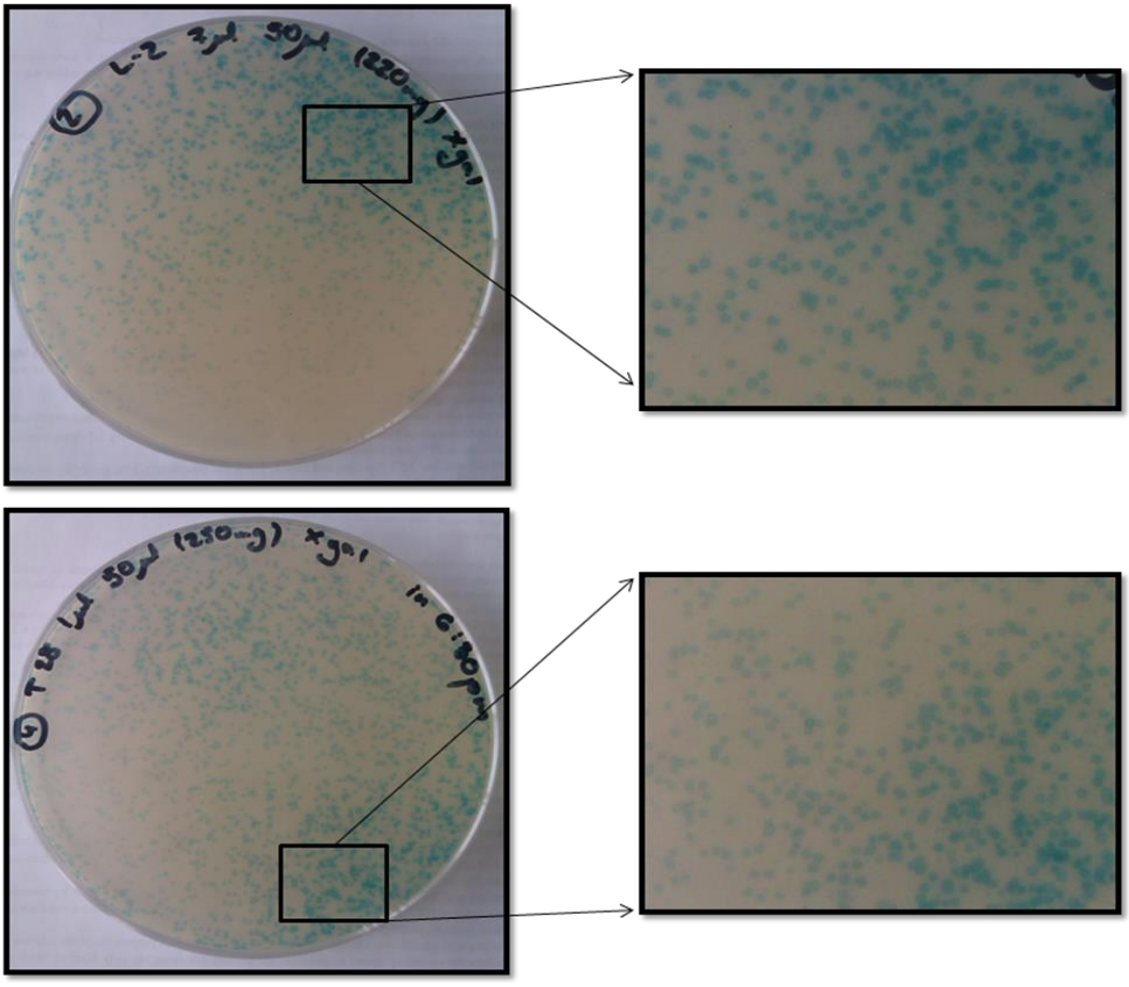


Figure 3.2 Blue/white screening for recombinant plaques. An aliquot of L2 (upper plate) and another of T28 (lower plate) cDNA libraries were used to infect XL-1 blue *E. coli* as in SEREX protocol. The plates were incubated overnight. It can be seen from Image 3.2 that all plaques on those two plates appeared blue – containing non-recombinant phage.

A PCR was set with samples of four cDNA libraries currently used in SEREX screening. PCR was carried as described earlier with T3 and T7 primer pair. As it can be seen from **Figure 3.3**, the primer pair did not amplify a product from none of the four libraries used as templates. The PCR reaction was repeated on a separate occasion with the same result.

Figure 3.3 PCR for presence of cDNA inserts within L1, L2, T27 and T28 cDNA libraries. The products of the PCR were loaded on a 1% agarose gel and gel electrophoresis was carried out for 45 minutes at 100V. The ladder used was HirepLadder I. No bands seen in lanes for L1, L2, T27 or T28.

3.2 cDNA testis library construction and amplification

Library was constructed and amplified according to the protocol provided by Stratagene. Subsequently, four NZY plates were prepared as in SEREX protocol with the addition of X-gal and incubated overnight to determine the titer.

3.2.1 Plating and titering of the primary cDNA library

The newly constructed library was used in Day 1 of SEREX protocol to confirm the recombinancy of the phages and also to determine its titer (plaque forming units per µl).

Table 3.1 Plating and titering the primary cDNA library on 150mm NZY plates

Plate No.	1	2	3	4
cDNA library added	1µl	1.5µl	2.5µl	3.5µl
Total number of plaques	33	52	84	109
Recombinant plaques	27	46	81	101
Non-recombinant plaques	6	6	3	8
Plaque forming units	**33 per µl**	**35 per µl**	**34 per µl**	**31 per µl**

The number of plaques weredivided by the amount of cDNA library added, to determine the plaque forming units (pfu) per µl. The primary library had a very low efficiency and therefore, the entire ligation was used to amplify the primary library.

3.2.2 Plating and titering of the amplified cDNA library

Following amplification (as outlined in Stratagene protocol), the newly amplified cDNA library was used in Day 1 of SEREX and eight NZY plates were prepared to determine the pfu per µl.

Table 3.2 Plating and titering the amplified cDNA library on 150mm NZY plates

Plate No.	1A	2A	3A	4A	1B	2B	3B	4B
cDNA library added	1µl of 1:10	5µl of 1:10	1µl of 1:50	1µl of 1:100	1µl of 1:10	5µl of 1:10	1µl of 1:50	1µl of 1:100
Total No. plaques	207	1015	42	19	216	1055	47	16
Recombinant plaques	169	864	39	18	193	924	43	16
Non-recombinant plaques	38	151	3	1	23	131	4	0
Plaque forming units (per µl)	**2070**	**2030**	**2100**	**1900**	**2160**	**2110**	**2350**	**1600**

Two sets of four plates were prepared with the use of two random aliquots of the amplified cDNA library – aliquot A (plates 1A to 4A) and aliquot B (plates 1B to 4B). Following overnight incubation the plaques were counted and the total amount of plaques was used to determine the pfu per µl, taking under account the appropriate dilution factor. Dilution of the cDNA library was applied to avoid overgrowth and to allow accurate counting of plaques. The average pfu per µl was determined to be 2040 (The sum of all pfu divided by eight plates). The titer of the amplified library had been increased in comparison with the titer prior to amplification.

3.2.3 Confirming the recombinancy of the amplified cDNA library

Recombinant plaques were selected at random from four NZY plates (**Figure 3.4**) and used as templates for a PCR. The products were analysed on a 1% Agarose gel (each sample mixed with loading buffer 1:6).

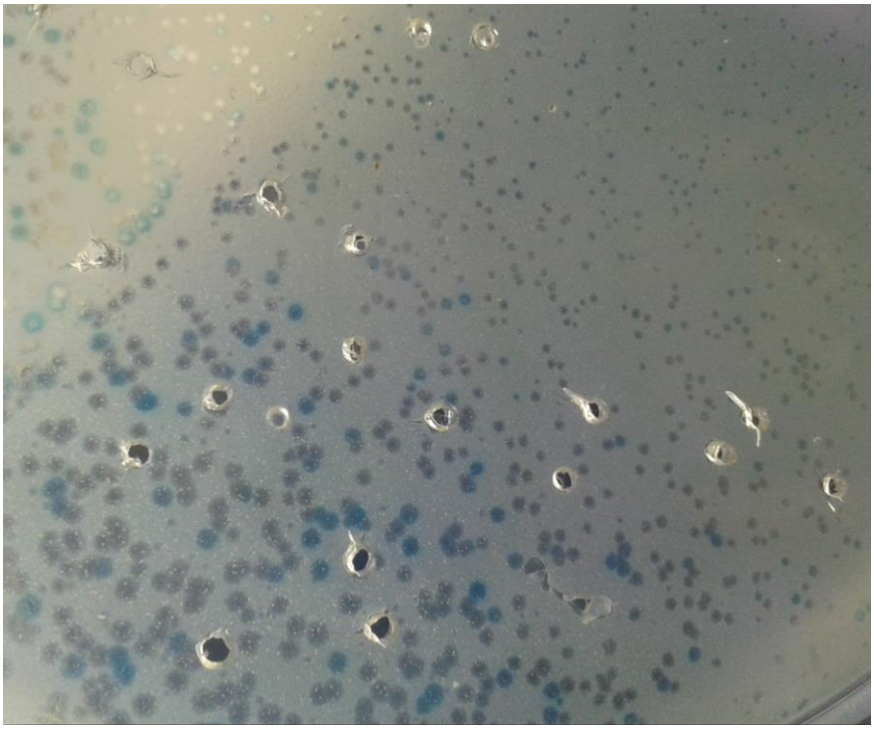


Figure 3.4 Selecting plaques from NZY plate at random for PCR to determine the range of cDNA inserts in the library. Each recombinant plaque was isolated with the use of a sterile glass Pasteur pipette and placed in the PCR mixture to serve as a template. The blue colour represents plaques containing non-recombinant phages (reaction of X-gal).

After eighty minutes at 90V, the 1% agarose gels containing the PCR samples were analysed under UV light. Images demonstrated that the randomly selected plaques contained cDNA inserts of different sizes and the library could now be aliquoted in smaller quantities and used for SEREX. The remaining cDNA testis library was processed as described before and stored at -80˚C for future use.

A **B**

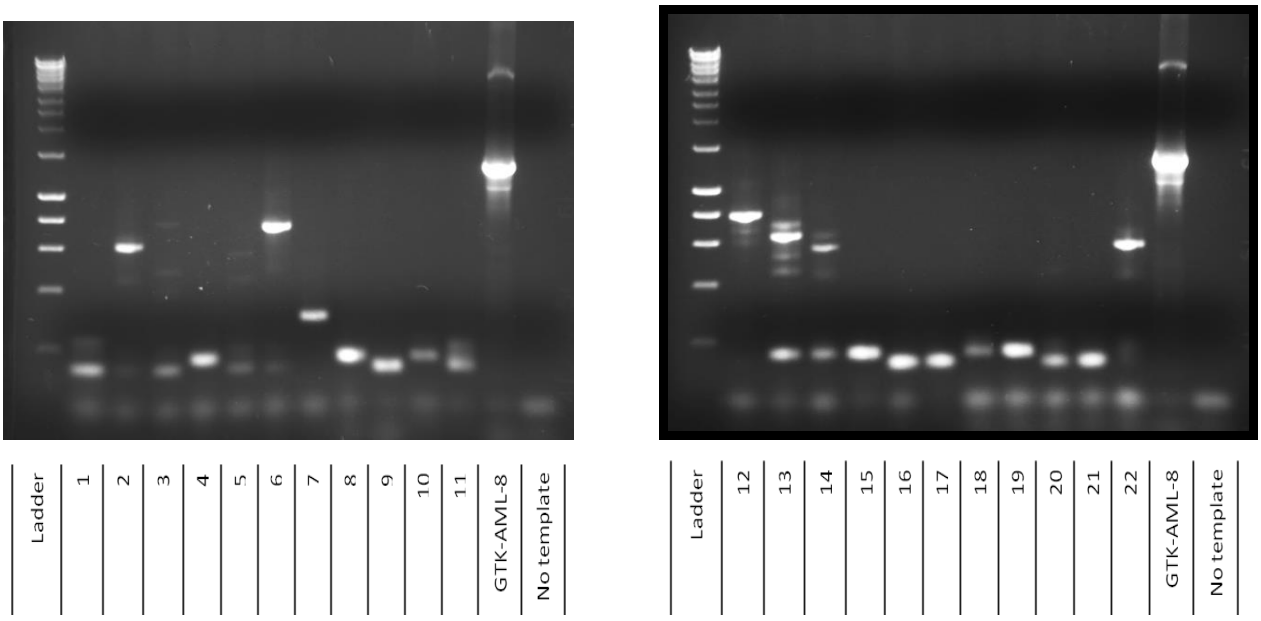


Figure 3.5 PCR analysis of the cDNA insert sizes in the testes cDNA library used for immunoscreening. Sample lanes 1 to 11 **(A)** and samples 12 to 22 **(B)** represent the amplified cDNA inserts from twenty two randomly selected plaques as amplified using the T3 and T7 primer pair. The ladder used was HyperLadder I. The last two lanes on each gel were loaded with a positive control – GTK-AML-8 (a cDNA insert described in Guinn *et al*, 2002 contained within the Invitrogen pBK-CMV plasmid) and a PCR reaction containing no template serves as a negative control. The bands were bright and clear, however some of the lanes contained secondary, faint bands which could be due to contamination which may have taken place during the isolation of the individual plaques. It could also be due to the close proximity of the plaques within the NZY agar.

CHAPTER 4 Results: Second round of immunoscreening following the SEREX technique

4.1 Secndary immunoscreening

Second round of primary immunoscreening was carried out as outlined in section 2.4. A detailed table of results can be found in **Appendix II**.

Three different patient sera were used throughout the primary immunoscreening of cDNA testis library with SEREX (**Table 4.1**). These were: CC005 (at 1:2000 and 1:200 dilutions), CC010 (at 1:200 dilution) and CC014 (at 1:200 dilution) and were all obtained from patients diagnosed with reactive CRC.

Table 4.1 Primary immunoscreening of testis cDNA library with SEREX.

Time frame	Serum ID:	No. of plaques screened	No. of potential positive plaques cored out	No. of confirmed positive plaques identified with that serum
29.1.2013 - 19.7.2013	**CC005**	**340,044**	**60**	**1**
	CC010	**190,612**	**10**	**0**
	CC014	**290,988**	**84**	**14**
Total No. of plaques:		**821,644**	**154**	**15**

Summary of the results obtained from primary immunoscreening with SEREX. With the aid of three sera (CC005, CC010 and CC014), 821 644 plaques were screened in total from a cDNA testis library in the course of six months.

4.2 Secondary immunoscreening with SEREX

Second round of secondary immunoscreening was carried out as outlined in section 2.5.

During primary immunoscreening with SEREX, there were 154 potential positive plaques that were cored out and stored for further investigations. Most of them (116 plaques) were used in secondary immunoscreening and fifteen of them were confirmed as immunoreactive plaques, containing a cDNA fragment that was capable of eliciting immune response (UOB-COL-1 to UOB-COL-15) (Table 4.2). Five of these antigens elicited immune response in two of the sera used (CC005 and CC014) and their sequence was mapped to four different genes: IGHG3, IGHG2, CYB5R3, RPL37A and SLC34A2 (as detailed in **Results Section 5**).

Following several uses of the sera and also optimisation of the reaction, the background noise on the membranes was significantly reduced. The more the sera were used, the easier it was to differentiate the positive from the negative plaques. Some of the immunoreactive plaques, identified through primary and secondary immunoscreening had a particularly definitive appearance (**Figure 4.1).**

It should be noted that some of the positive plaques that are recognised through primary or secondary immunoscreening do not appear as a clear spot on the membrane but rather as a darker ring surrounding a plaque that is negative in appearance. These should not be disregarded and should be isolated and confirmed through secondary immunoscreening.

Table 4.2 Secondary immunoscreening – confirmed antigens.

Name	Plaque ID	Serum identified with:	Confirmed with:	Confirmation date	Copies	Identified as (see section 5.1):
UOB-COL-1	060213-2-1	CC014	CC014	19/02/2013	3	**IGHG3 gene**
			CC005	19/02/2013	3	**IGHG3 gene**
UOB-COL-2	190213-5-1	CC014	CC014	28/02/2013	3	**IGHG2 gene**
UOB-COL-3	050313-5-5	CC014	CC005	14/03/2013	3	*UNIDENTIFIED*
			CC005	27/04/2013	4	*UNIDENTIFIED*
UOB-COL-4	050313-6-1	CC014	CC005	21/03/2013	3	**CYB5R3 gene**
			CC014	07/04/2013	3	
			CC005	29/04/2013	4	
UOB-COL-5	050313-6-8	CC014	CC005	21/03/2013	3	*UNIDENTIFIED*
			CC014	29/04/2013	3	**SLC34A2 gene**
			CC005	29/04/2013	5	*UNIDENTIFIED*
UOB-COL-6	050313-6-6	CC014	CC005	21/03/2013	3	*UNIDENTIFIED*
			CC014	07/04/2013	3	
UOB-COL-7	050313-7-6	CC014	CC005	21/03/2013	3	*UNIDENTIFIED*
UOB-COL-8	190213-3-1	CC005	CC005	07/04/2013	8	*UNIDENTIFIED*
UOB-COL-9	050313-7-1	CC014	CC005	11/04/2013	5	*UNIDENTIFIED*
UOB-COL-10	050313-6-9	CC014	CC005	11/04/2013	5	*UNIDENTIFIED*
UOB-COL-11	050313-5-3	CC014	CC005	11/04/2013	5	**RPL37A gene**
UOB-COL-12	050313-6-7	CC014	CC005	11/04/2013	5	*UNIDENTIFIED*
UOB-COL-13	050313-7-5	CC014	CC005	11/04/2013	5	*UNIDENTIFIED*
UOB-COL-14	050313-4-5	CC014	CC005	14/06/2013	3	*UNIDENTIFIED*
UOB-COL-15	050313-5-2	CC014	CC005	14/06/2013	3	*UNIDENTIFIED*

Outline of the antigens, confirmed through secondary immunoscreening with SEREX and the outcome of their sequencing. UOB-COL-1 and UOB-COL-2 appear to have similar properties and map to the same gene IGHG2 (also a copy of UOB-COL-1 confirmed with CC014 is mapped to IGHG3 gene). The sequence encoded by the cDNA of UOB-COL-4 appeared to be part of CYB5R3 gene and the one belonging to UOB-COL-11 – part of RPL37A. Also, the sequence belonging to UOB-COL-5 appeared to be part of the SLC34A2 gene.

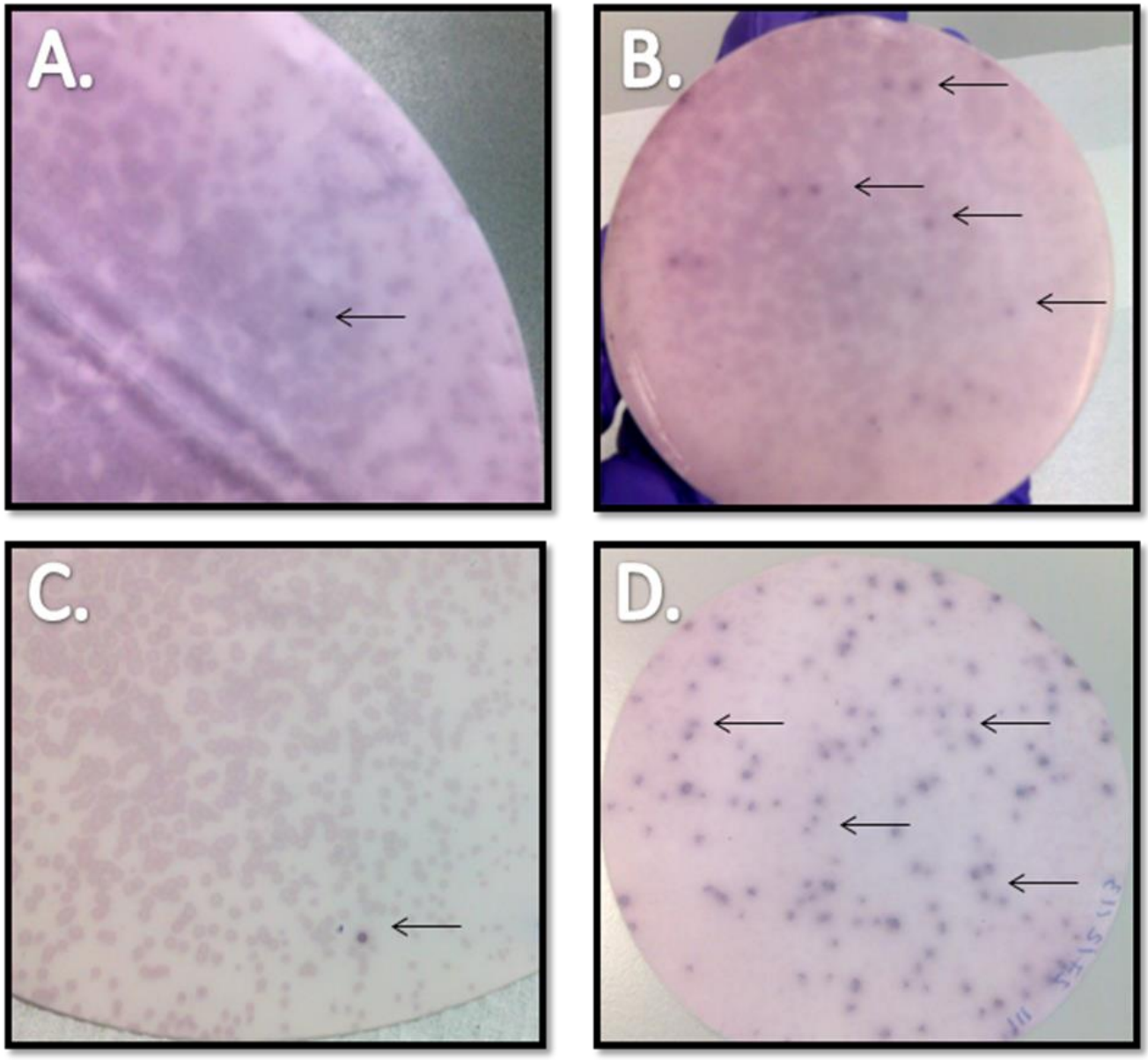


Figure 4.1 UOB-COL-1 and UOB-COL-2 in primary and secondary immunoscreening with SEREX. (A) The primary identification of a positive plaque with CC014 (arrow on A), which was isolated and labelled as 060213-2-1 (the date – 06.02.13; the membrane number – 2; the plaque number – 1). **(B)** The same plaque was then rescreened via secondary immunoscreening with SEREX and was confirmed to be immunoreactive as it was recognised by CC014 (arrows on B.). Three of these positive plaques were isolated and labelled as UOB-COL-1-c1 to c3 (UOB-COL-1 – name of the antigen; c1 – plaque number). **(C)** The primary identification of a positive plaque with CC014 (arrow on C.), which was isolated and labelled 190213-5-1 (date; membrane number; plaque number). **(D)** The same plaque was them rescreened via secondary immunoscreening with SEREX and was confirmed to be immunoreactive as it was recognised by CC014 (arrows on D.). Three of these plaques were isolated and labelled as UOB-COL-2-c1 to c3 (name of antigen; plaque number).

4.3 Tertiary immunoscreening with SEREX

Tertiary immunoscreening was carried out as outlined in section 2.7. As the sera obtained from patients with immunoreactive CRC (CC005, CC010 and CC014) that were used in SEREX were now a few months old, the results obtained from tertiary immunoscreening were inconclusive. Further investigations and a repeat of tertiary immunoscreening should be carried out. The results from the tertiary immunoscreening of confirmed antigens with CRC sera (CC005, CC010 and CC014) are shown in **Figure 4.2** and sera from eight healthy volunteers as shown in **Figure 4.3** are summarised in **Table 4.3**.

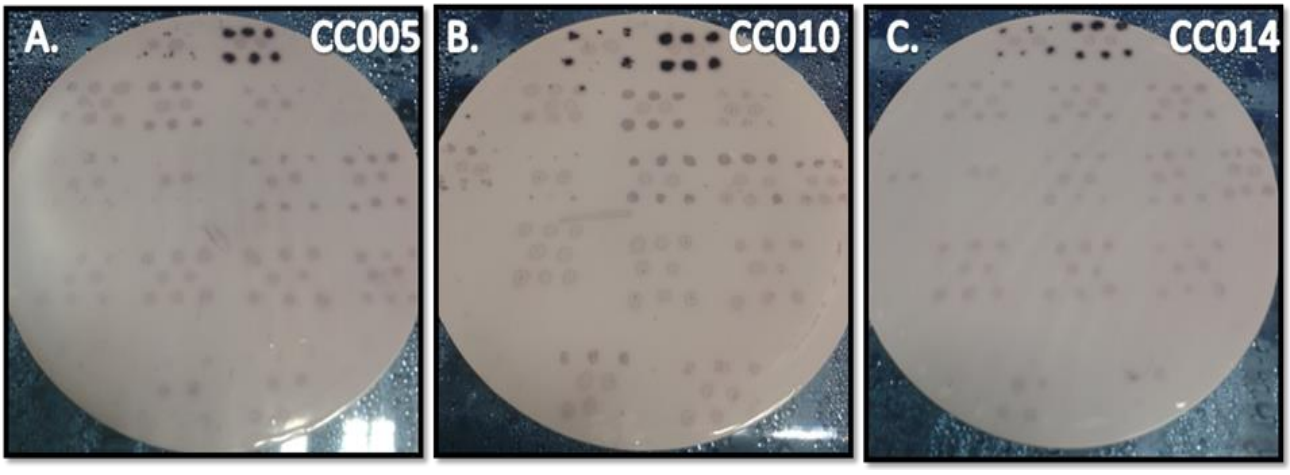


Figure 4.2 Tertiary immunoscreening of UOB-COL-1 to UOB-COL-15 with CRC sera. Each membrane contains protein products, produced by cDNA phage belonging to antigens UOB-COL-1 to UOB-COL-15. The two spots in the middle of each section represent negative controls

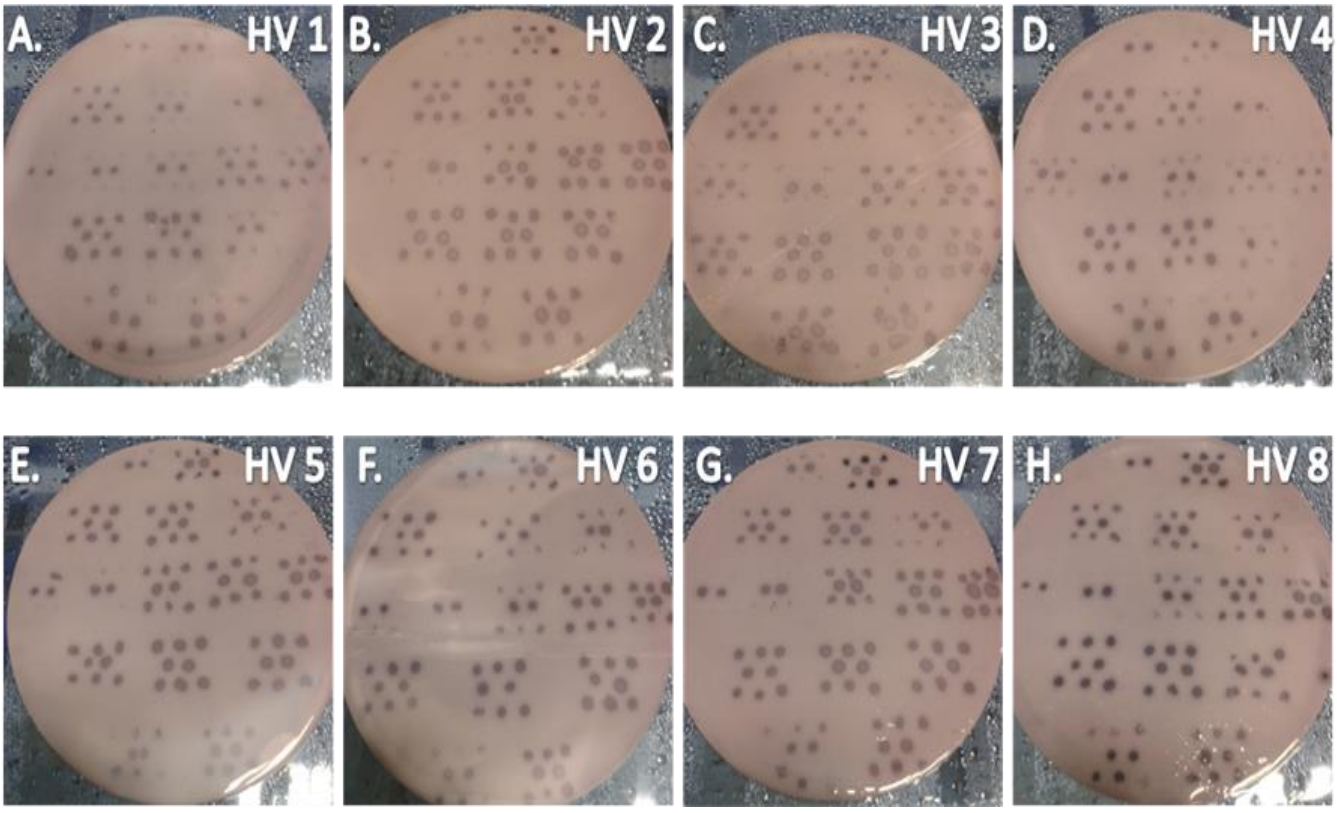


Figure 4.3 Tertiary screening of UOB-COL-1 to UOB-COL-15 with healthy volunteer sera. Each membrane contains protein products, produced by cDNA phage belonging to antigens UOB-COL-1 to UOB-COL-15. The two spots in the middle of each section represent negative controls. Membranes were treated with 8 different sera obtained from healthy volunteers.

Table 4.3 Summary of results from tertiary immunoscreening of each cDNA with sera from Dukes' B reactive and non-reactive patients and healthy donors.

	Dukes B reactive patients			Healthy donors (n=8)
Antigen id/name	CC005	CC010	CC014	
UOB-COL-1 **IGHG3**	√	-	√	Recognised by HV-2; 3, 5, 6, 7, 8
UOB-COL-2 IGHG2	-	-	√	Not recognised

UOB-COL-3 Unidentified	√	-	√	Recognised by HV-2, 5, 6, 7, 8.
UOB-COL-4 **CYB5R3 gene**	√	-	√	Recognised by HV-2, 3, 4, 5, 6, 7, 8.
UOB-COL-5 **SLC34A2**	√	-	√	Recognised by all
UOB-COL-6 Unidentified	√	-	√	Recognised by HV-1, 2, 3, 5, 6, 7, 8
UOB-COL-7 Unidentified	√	-	√	Recognised by HV-1, 2, 3, 5, 6, 7, 8
UOB-COL-8 Unidentified	√	-	-	Recognised by HV-2, 4, 5, 6, 7, 8
UOB-COL-9 Unidentified	√	-	√	Not recognised
UOB-COL-10 Unidentified	√	-	√	Recognised by HV- 3 and 4
UOB-COL-11 **RPL37A**	√	-	√	Recognised by HV-2, 3, 5, 6, 7, 8
UOB-COL-12 Unidentified	√	-	√	Recognised by all.
UOB-COL-13 Unidentified	√	-	√	Recognised by all
UOB-COL-14 Unidentified	√	-	√	Recognised by all
UOB-COL-15 Unidentified	√	-	√	Recognised by all
Total number of antigens recognised	14/15	**0/15**	14/15	

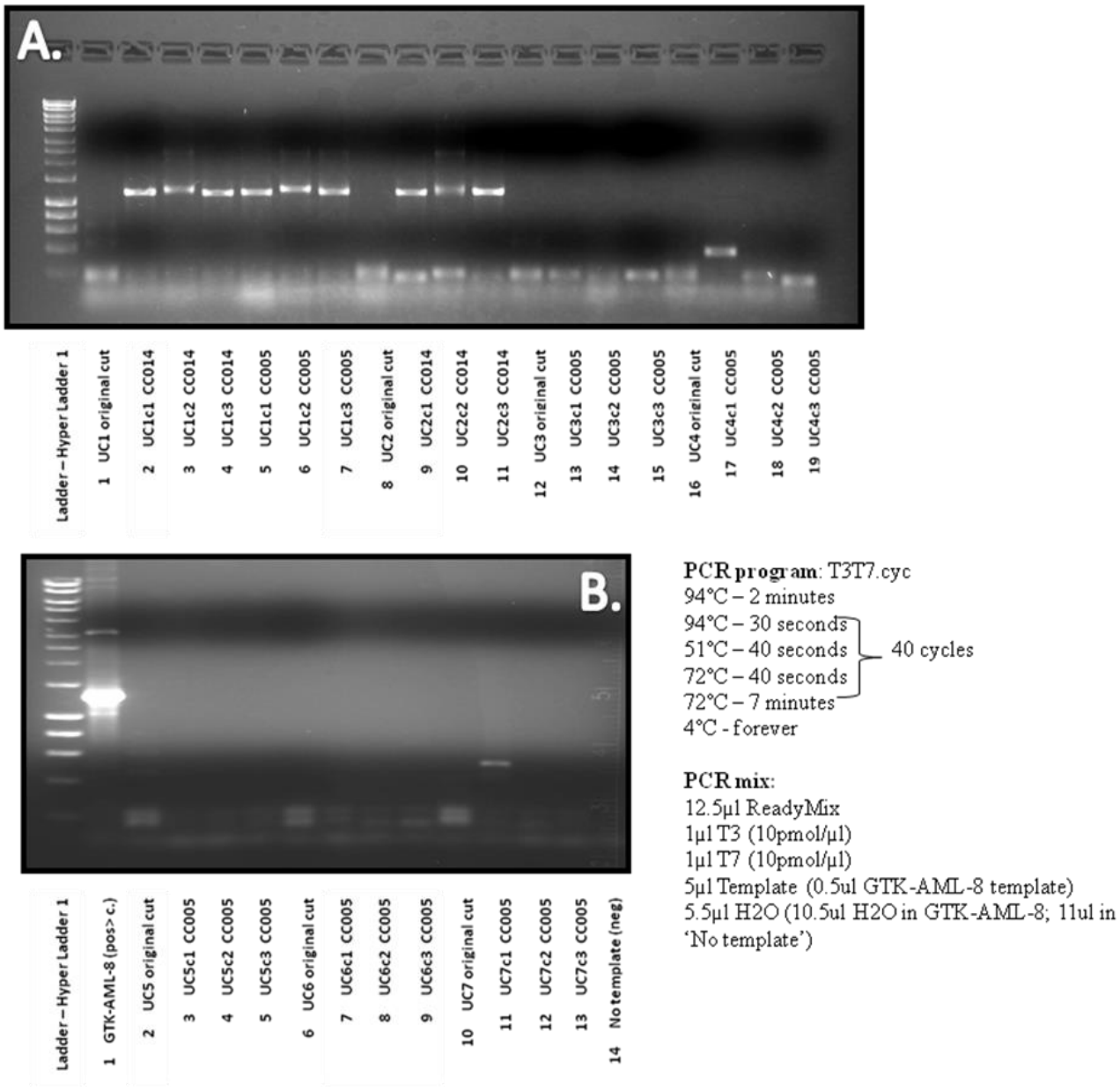
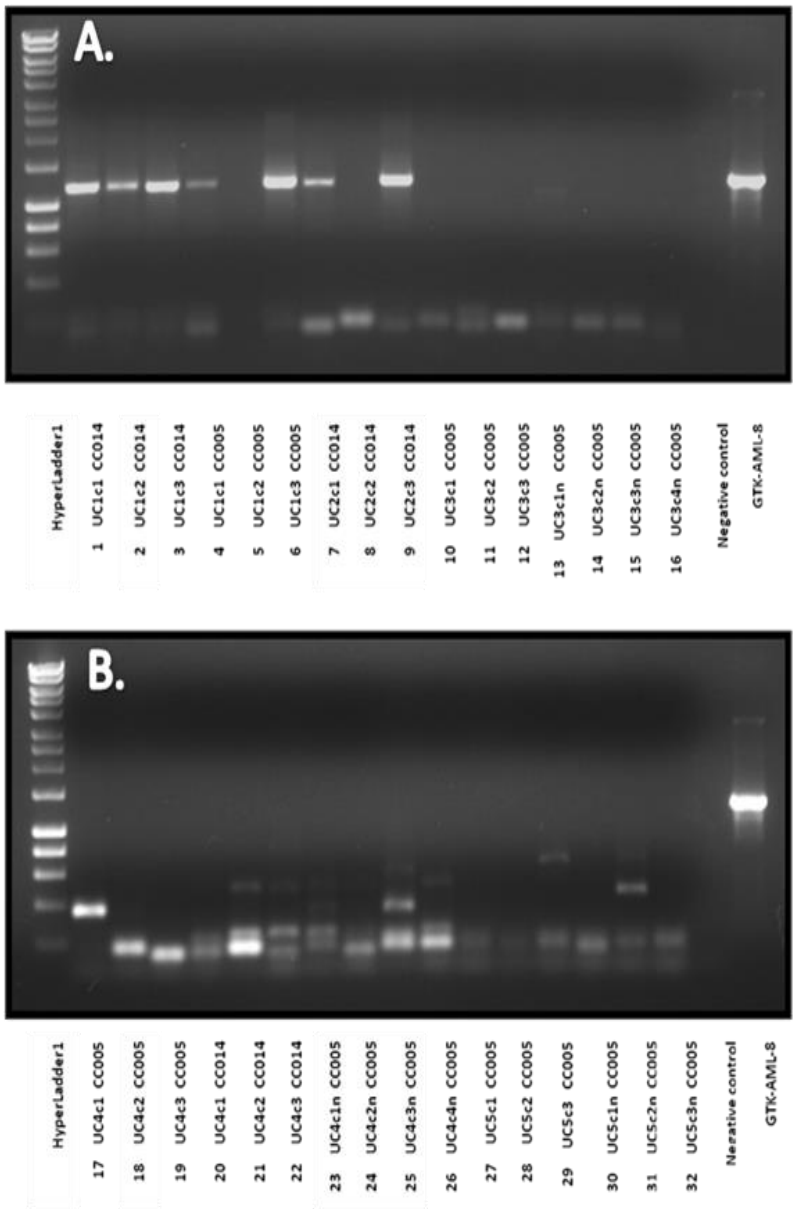


Figure 4.4 UOB-COL-1 to UOB-COL-7 analysed via PCR. (A) and (B) 1% agarose gels as seen under UV light loaded with the products of a PCR reaction. The products are amplified cDNA inserts contained within the isolated copies of the seven confirmed immunoreactive plaques to that date. The gel was run to confirm the presence of inserts within the positive phage and also the size of the insert itself. Products of UOB-COL-1 and UOB-COL-2 seemed to be of a similar size. UC1* - refers to UOB-COL-1 (original stock isolated from primary screening. UC1c1* refers to UOB-COL-1, copy (plaque) 1 and so on.

CHAPTER 5 Results: Sequencing and antigen identification

5.1 PCR of confirmed antigens and preparation for sequencing

The immunoreactive cDNA inserts were amplified and isolated via PCR. The products were gel cleaned and the quality and quantity of the isolated DNA were analysed with Nanodrop 2000. The products were then processed as described in section 2.8 (Material and Methods) and sent off for sequencing. First lot of antigens (UOB-COL-1 to UOB-COL-5) as seen in **Figure 5.1**:



CUT OUT FOR SEQUENCING:
1, 2, 3, 4, 6, 7, 8, 9, 12, 13, 15, 17, 18, 19, 21 (large), 21 (small), 22 (middle), 25 (middle), 29 (large), 29 (small), 30, 31 (large), 31 (small), 32 (large) – gel clean up and sequencing.

PCR program: T3T7.cyc
94°C – 2 minutes
94°C – 30 seconds
51°C – 40 seconds
72°C – 40 seconds } 40 cycles
72°C – 7 minutes
4°C - forever

PCR mix:
12.5µl ReadyMix
1µl T3 (10pmol/µl)
1µl T7 (10pmol/µl)
5µl Template (0.5ul GTK-AML-8 template)
5.5µl H2O (10.5ul H2O in GTK-AML-8; 11ul in 'No template')

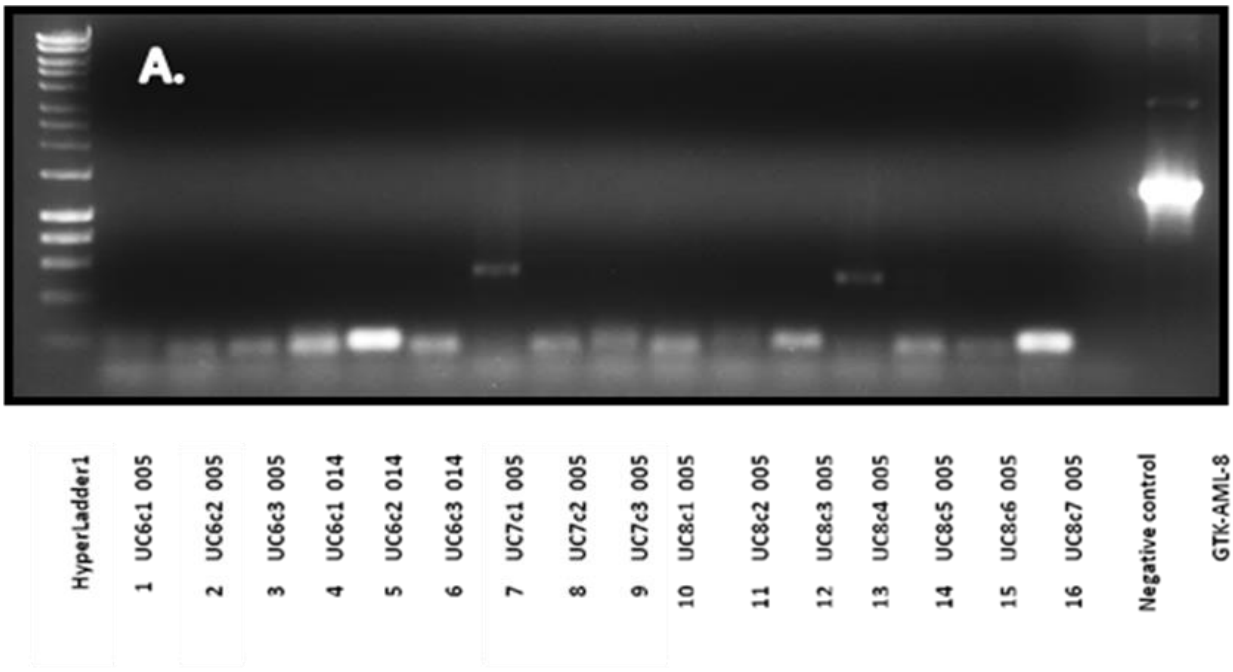
Figure 5.1 PCR of UOB-COL-1 to UOB-COL-5 in preparation for sequencing. PCR products are loaded on a 1% agarose gel. Following observation, the required bands were cut out of the gel and placed in 1.5ml sterile eppendorf tubes to undergo gel clean up and nanodrop reading prior to being sent for sequencing.

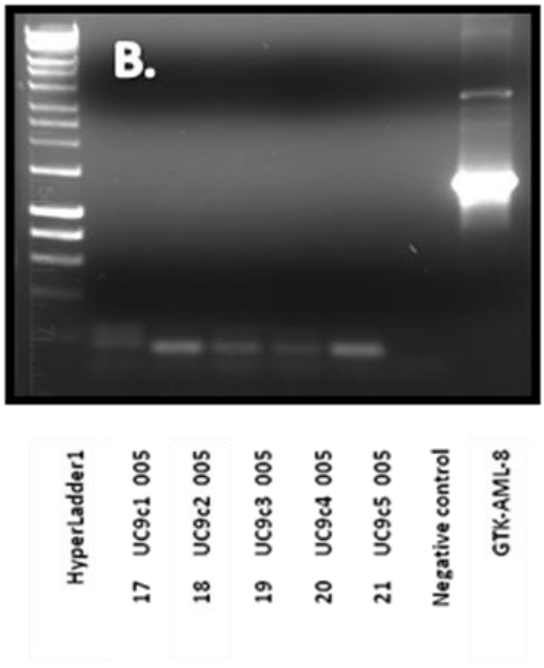
After the samples were isolated from the gel and cleaned up, a Nanodrop analisys was carried out to confirm the quantity and the quality of the DNA within each sample (**Table 5.1**).

Table 5.1 Nanodrop readings for products of UOB-COL-1 to UOB-COL-5

Sample ID	Date	Nucleic Acid Conc.	Unit	A260	A280	260/ 280	260/ 230	Sample Type	Factor
Blank	10/06/13	**0.1**	**ng/µl**	0.003	0.012	**0.24**	0.19	DNA	50
1 UC1-1 14	10/06/13	**3**	**ng/µl**	0.059	0.036	**1.64**	0.03	DNA	50
2 UC1-2 14	10/06/13	**4**	**ng/µl**	0.079	0.05	**1.58**	0.02	DNA	50
3 UC1-3 14	10/06/13	**6**	**ng/µl**	0.119	0.079	**1.51**	0.06	DNA	50
4 UC1-3 14	10/06/13	**2.1**	**ng/µl**	0.042	0.022	**1.91**	0.02	DNA	50
6 UC1-3 05	10/06/13	**5.1**	**ng/µl**	0.101	0.069	**1.48**	0.05	DNA	50
7 UC2-1 14	10/06/13	**2.7**	**ng/µl**	0.054	0.019	**2.81**	0.02	DNA	50
8 UC2-2 14	10/06/13	**5.2**	**ng/µl**	0.104	0.061	**1.71**	0.02	DNA	50
9 UC2-3 014	10/06/13	**5.2**	**ng/µl**	0.104	0.058	**1.79**	0.03	DNA	50
12 UC3-1 05	10/06/13	**4**	**ng/µl**	0.079	0.044	**1.8**	0.02	DNA	50
13 UC3-1 NEW 05	10/06/13	**2**	**ng/µl**	0.039	0.018	**2.17**	0.01	DNA	50
15 UC3-3 NEW 05	10/06/13	**4**	**ng/µl**	0.081	0.054	**1.5**	0.02	DNA	50
17 UC4-1 05	10/06/13	**5.6**	**ng/µl**	0.113	0.065	**1.74**	0.06	DNA	50
18 UC4-2 05	10/06/13	**3.2**	**ng/µl**	0.063	0.04	**1.58**	0.02	DNA	50
19UC4-3 05	10/06/13	**3**	**ng/µl**	0.06	0.032	**1.87**	0.01	DNA	50
21 UC4-2 014 BIG	10/06/13	**3.2**	**ng/µl**	0.064	0.035	**1.83**	0.02	DNA	50
21 UC4-2 014 SM	10/06/13	**3.6**	**ng/µl**	0.072	0.034	**2.15**	0.03	DNA	50
22 UC4-3 014 MID	10/06/13	**3.8**	**ng/µl**	0.075	0.044	**1.71**	0.02	DNA	50
25 UC4-3 05 NEW MID	10/06/13	**4.5**	**ng/µl**	0.09	0.054	**1.65**	0.03	DNA	50
29 UC5-3 05 BIG	10/06/13	**2.5**	**ng/µl**	0.049	0.033	**1.51**	0.02	DNA	50
29 UC5-3 05 SM	10/06/13	**3.2**	**ng/µl**	0.063	0.043	**1.47**	0.02	DNA	50
30 UC5-1 NEW 05	10/06/13	**3.3**	**ng/µl**	0.065	0.03	**2.17**	0.02	DNA	50
31 UC5-2 NEW BIG	10/06/13	**4.4**	**ng/µl**	0.089	0.051	**1.73**	0.03	DNA	50
31 UC5-2 NEW SM	10/06/13	**3.8**	**ng/µl**	0.076	0.052	**1.45**	0.04	DNA	50
32 UC5-3 NEW BIG	10/06/13	**3.4**	**ng/µl**	0.067	0.045	**1.51**	0.03	DNA	50

The desired samples were processed as described in section 2.8 and send off for sequencing. The concentration of DNA within the samples was lower than expected and it was difficult to process most of the samples for sequencing. The following time, PCR samples are to be used as templates for a secondary PCR reaction to attempt to increase the concentration of DNA within each sample.





PCR program: T3T7.cyc
94°C – 2 minutes
94°C – 30 seconds
51°C – 40 seconds } 40 cycles
72°C – 40 seconds
72°C – 7 minutes
4°C - forever

PCR mix:
12.5µl ReadyMix
1µl T3 (10pmol/µl)
1µl T7 (10pmol/µl)
5µl Template (0.5ul GTK-AML-8 template)
5.5µl H2O (10.5ul H2O in GTK-AML-8; 11ul in 'No template')

CUT OUT FOR SEQUENCING:
3, 4, 5, 7, 8, 12, 13, 16, 18, 21 – gel clean up and sequencing.

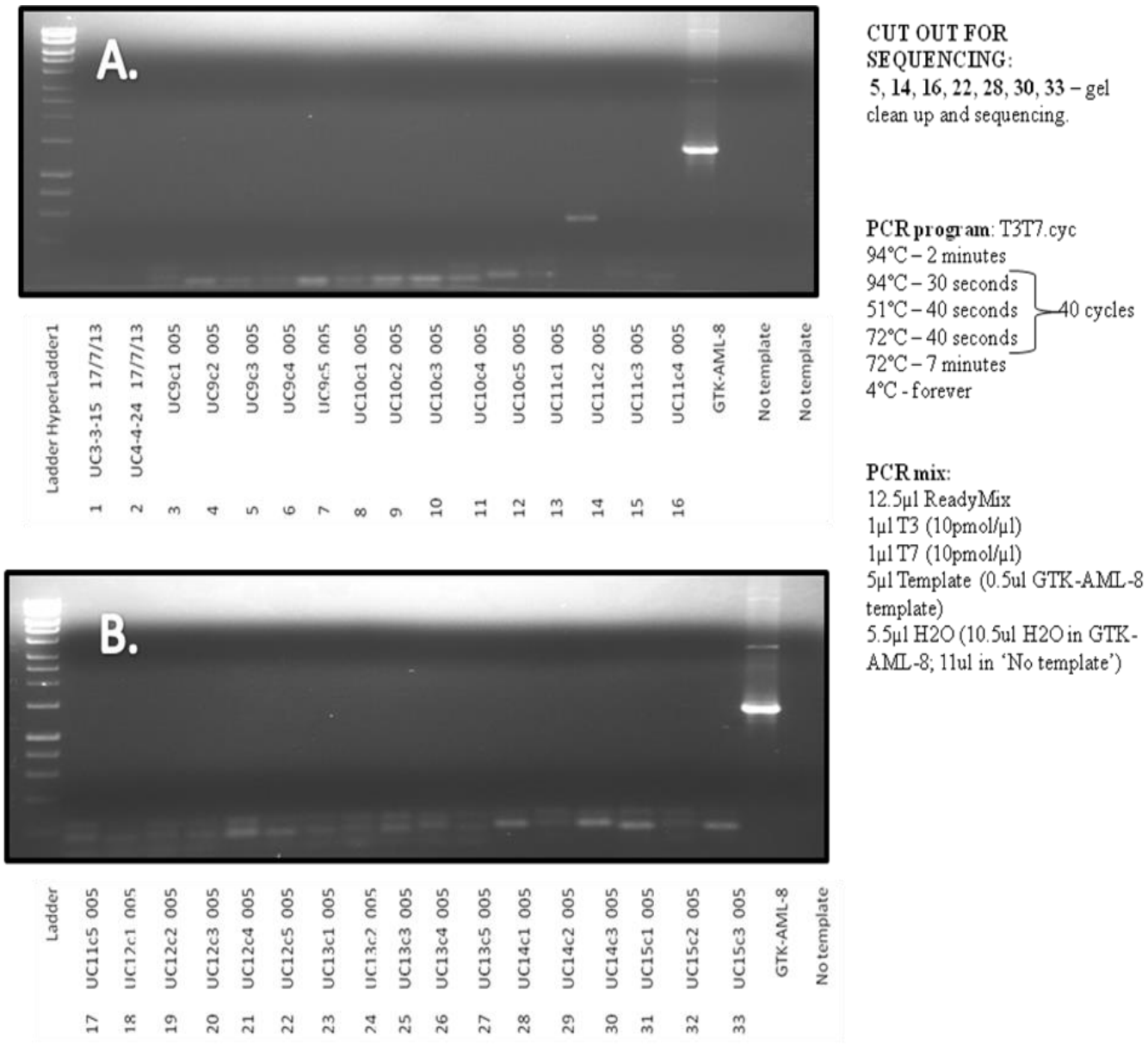
Figure 5.2 PCR of UOB-COL-6 to UOB-COL-9. PCR products are loaded on a 1% agarose gel. Following observation, the required bands were cut out of the gel and placed in 1.5ml sterile eppendorf tubes to undergo gel clean up and nanodrop reading prior to being sent for sequencing.

Table 5.2 Nanodrop readings of UOB-COL-6 to UOB-COL-9.

Sample ID	Date	Nucleic Acid Conc.	Unit	A260	A280	260/ 280	260/ 230	Sample Type	Factor
Blank	10/06/13	**0.1**	**ng/µl**	0.003	0.016	**0.19**	0.35	DNA	50
3 UC6-3 05	10/06/13	**6.8**	**ng/µl**	0.136	0.089	**1.53**	0.06	DNA	50
4 UC6-1 014	10/06/13	**8.5**	**ng/µl**	0.17	0.096	**1.77**	0.02	DNA	50
5 UC6-2 014	10/06/13	**13.7**	**ng/µl**	0.275	0.149	**1.85**	0.03	DNA	50
7 UC7-1 05	10/06/13	**6.7**	**ng/µl**	0.133	0.079	**1.69**	0.03	DNA	50
8 UC7-2 05	10/06/13	**6.8**	**ng/µl**	0.137	0.073	**1.87**	0.02	DNA	50
12 UC8-3 05	10/06/13	**6.1**	**ng/µl**	0.123	0.062	**1.98**	0.01	DNA	50
13 UC8-4 05	10/06/13	**7.8**	**ng/µl**	0.155	0.085	**1.82**	0.02	DNA	50
16 UC8-7 05	10/06/13	**11.8**	**ng/µl**	0.236	0.147	**1.6**	0.05	DNA	50
18 UC9-2 05	10/06/13	**6.8**	**ng/µl**	0.136	0.083	**1.64**	0.05	DNA	50
21 UC9-5 05	10/06/13	**9.2**	**ng/µl**	0.184	0.109	**1.69**	0.03	DNA	50

Products were analysed on a 1% agarose gel. Selected bands were cut out, gel cleaned and processed further for sequencing.

The desired samples were processed and send off for sequencing. The concentration of cDNA qas fairly improved in comparison to the data shown in **Table 5.1**. However, further improvements need to take place to increase the product yield following PCR and gel clean up.



CUT OUT FOR SEQUENCING:
5, 14, 16, 22, 28, 30, 33 – gel clean up and sequencing.

PCR program: T3T7.cyc
94°C – 2 minutes
94°C – 30 seconds
51°C – 40 seconds
72°C – 40 seconds
} 40 cycles
72°C – 7 minutes
4°C - forever

PCR mix:
12.5µl ReadyMix
1µl T3 (10pmol/µl)
1µl T7 (10pmol/µl)
5µl Template (0.5ul GTK-AML-8 template)
5.5µl H2O (10.5ul H2O in GTK-AML-8; 11ul in 'No template')

Figure 5.3 PCR of UOB-COL-9 to UOB-COL-15. Products were analysed on a 1% agarose gel. Selected bands were cut out, gel cleaned and processed further for sequencing. Most of the products were small (around 100bp) which was inconclusive of whether this is the size of the product or a remaining of unused primers. Selected samples were isolated and analysed with a Nanodrop.

Table 5.3 Nanodrop readings for products of UOB-COL-9 to UOB-COL-15

Sample ID	Date	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
blank	05/08/13	**0.2**	ng/µl	0.004	0.01	**0.43**	-0.41	DNA	50
5 UC9c3 005	05/08/13	**1.5**	ng/µl	0.03	0.015	**1.96**	0.01	DNA	50
14 UC11c2 005	05/08/13	**5.8**	ng/µl	0.117	0.061	**1.93**	0.03	DNA	50
16 UC11c4 005	05/08/13	**1.5**	ng/µl	0.03	0.018	**1.65**	0.01	DNA	50
22 UC12c5 005	05/08/13	**1.7**	ng/µl	0.034	0.029	**1.17**	0.03	DNA	50
28 UC14c1 005	05/08/13	**2.6**	ng/µl	0.052	0.038	**1.37**	0.03	DNA	50
30 UC14c3 005	05/08/13	**3.9**	ng/µl	0.077	0.042	**1.86**	0.02	DNA	50
32 UC15c3 005	05/08/13	**4.3**	ng/µl	0.085	0.052	**1.64**	0.03	DNA	50

Results of sequencing were analysed and interpretations made appropriately (**Section 5.2**).

5.2 Sequencing and sequence interpretations

The sequences of the analysed cDNAs were analysed as described in **Section 2.8**. This analysis led to the following findings:

5.2.1 IGHG3 gene

Gene Description: **IGHG3**; Immunoglobulin heavy constant gamma 3 (G3m marker) located on chromosome 14 (Image 3.1).
Location: Chromosome 14: 106,235,439-106,237,742 reverse strand.
Transcripts: This gene has one transcript (splice variant).
Gene type: Known IG C gene.
Prediction method: Immunoglobulin (Ig) and T-cell receptor (TcR) annotation imported from the manually curated IG gene set from the Havana project. (It corresponds to Havana gene OTTHUMG00000152539 (version 1)).

5.2.1.1 UOB-COL-1 clone 1 (serum CC014) identified as a transcript of IGHG3 gene

Sequence fragment used for identification (T3 primer reading):

CTNCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGGCTGCAGGAATTCGGCACGAGGGTGGAACTCAGGCGCTCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCT
ACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGACCTCCAGCAACTTCGGCACCCAGACCTACACCTGCAACGTAGATCACAAGCCCAGCAACACCAAGGTGGAC
AAGACAGTTGAGCGCAAATGTTGTGTCGAGTGCCCACCGTGCCCAGCACCACCTGTGGCAGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCG
GACCCCTGAGGTCACGTGCGTGGTGGTGGACGTGAGCCACGAAGACCCCGAGGTCCAGTTCAACTGGTACGTGGACGGCATGGAGGTGCATAATGCCAAGACAAAGCCACGGGA
GGAGCAGTTCAACAGCACGTTCCGTGTGGTCAGCGTCCTCACCGTCGTGCACCAGGACTGGCTGAACGGNAAGGAGTACAAGTGCAAGGTCTCCAACAAAGGCCTCCCAGCCNCC
ATCGAGAAAACCATCTCCAAAANCAAAGGGCAGCCCCGAGAACCACAGGTGTACANCCTGCCCCCATTCCGGGAGGAGATGACNAATAACCATNTCAGCCTGANTTTACCTGGT
NAAAGGNTTTNTACNCNNANCAACATTATTNTTGTANTNGGAGAGTAATTTGGCAATTNNTTAAATAATTATANATNANTACAANCTCTAA

Fragment matched on Ensemble Genome Browser:

>chromosome: **14:106235693:106236607-1**

CTCCAGCTCAAGGCGGGACAAGAGCCCTAGAGTGGCCTGAGTCCAGGGACAGGCCCCAGCAGGGTGCTGACGCATCCACCTCCATCCC
AGATCCCCGTAACTCCCAATCTTCTCTCTGCAGAGCCCAAATCTTGTGACACACCTCCCCCGTGCCCAAGGTGCCCAGGTAAGCCAGCCC
AGGCCTCGCCCTCCAGCTCAAGGCAGGACAGGTGCCCTAGAGTGGCCTGCATCCAGGGACAGGTCCCAGTCGGGTGCTGACACATCTGC
CTCCATCTCTTCCTCAG**CACCTGAACTCCTGGGAGGACCGTCAGTCTTCCTCTTCCCCCCAAAAACCCAAGGATACCCTTATGATTTC
CCGGACCCCTGAGGTCACGTGCGTGGTGGTGGACGTGAGCCACGAAGACCCCGAGGTCCAGTTCAAGTGGTACGTGGACGGCG
TGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTTCCGTGTGGTCAGCGTCCTCACCGTCCTG
CACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTC
CAAAACCAAAGG**TGGGACCCGCGGGGTATGAGGGCCACATGGACAGAGGCCAGCTTGACCCACCCTCTGCCCTGGGAGTGACCGCTGT
GCCAACCTCTGTCCCTACAGGA**CAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGAGGAGATGACCAAGAACCAG
GTCAGCCTGA**CCTG**CCTGGTCAAAGGCTT**CTACCCCAGCGACATCGCCGTGGAGTGGGAGAGCAGCGGGCAGCCGGAGAACAACTA
CAACACCACGCCTCCCATGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCA

***THIS STYLE:** Location of selected alignment
***THIS STYLE:** Location of other alignments
*THIS STYLE: Location of Exons

Alignment information:

Query location:	unnamed	**290** to **604** (-)
Database location:	14	106235993 to 106236307 (+)
Genomic location:	14	106235993 to 106236307 (+)
Alignment score:	1499	
E-value:	1.1e-257	
Alignment length:	**315**	
Percentage identity:	**96.19%**	

5.2.1.2 UOB-COL-1 clone 3 (serum CC014) identified as a transcript of IGHG3 gene

Sequence fragment used for identification (T3 primer reading):

TAACTCCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGGGTGGAACTCAGGCGCTCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTAC
AGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGACCTCCAGCAACTTCGGCACCCAGACCTACACCTGCAACGTAGATCACAAGCCCAGCAACACCAAGGTGGACAAGAC
AGTTGAGCGCAAATGTTGTGTCGAGTGCCCACCGTGCCCAGCACCACCTGTGGCAGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTG
AGGTCACGTGCGTGGTGGTGGACGTGAGCCACGAAGACCCCGAGGTCCAGTTCAACTGGTACGTGGACGGCATGGAGGTGCATAATGCCAAGACAAAGCCACGGGAGGAGCAGTTCA
ACAGCACGTTCCGTGTGGTCAGCGTCCTCACCGTCGTGCACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGGCCTCCCAGCCCCCATCGAGAAAACCAT
CTCCAAAACCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGAGGAGATGACCAANAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGNTTC

Fragment matched on Ensemble Genome Browser:

>chromosome **14:106235693:106236607-1**

CTCCAGCTCAAGGCGGGACAAGAGCCCTAGAGTGGCCTGAGTCCAGGGACAGGCCCCAGCAGGGTGCTGACGCATCCACCTCCATCCC
AGATCCCCGTAACTCCCAATCTTCTCTCTGCAGAGCCCAAATCTTGTGACACACCTCCCCCGTGCCCAAGGTGCCCAGGTAAGCCAGCCC
AGGCCTCGCCCTCCAGCTCAAGGCAGGACAGGTGCCCTAGAGTGGCCTGCATCCAGGGACAGGTCCCAGTCGGGTGCTGACACATCTGC
CTCCATCTCTTCCTCAGCACCTGAACTCCTGGG**AGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGATACCCTTATGATTTC**
CCGGACCCCTGAGGTCACGTGCGTGGTGGTGGACGTGAGCCACGAAGACCCCGAGGTCCAGTTCAAGTGGTACGTGGACGGCG
TGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTTCCGTGTGGTCAGCGTCCTCACCGTCCTG
CACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTC
CAAAACCAAAGGTGGGACCCGCGGGGTATGAGGGCCACATGGACAGAGGCCAGCTTGACCCACCCTCTGCCCTGGGAGTGACCGCTGT
GCCAACCTCTGTCCCTACAGGA**CAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGAGGAGATGACCAAGAACCAG**
GTCAGCCTGACCTGCCTGGTCAAAGGCTTCTACCCCAGCGACATCGCCGTGGAGTGGGAGAGCAGCGGGCAGCCGGAGAACAACTA
CAACACCACGCCTCCCATGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCA

***THIS STYLE:** Location of selected alignment
***THIS STYLE:** Location of other alignments
*THIS STYLE: Location of Exons

Alignment information:

Query location:	unnamed	**292** to **606** (-)
Database location:	14	106235993 to 106236307 (+)
Genomic location:	14	106235993 to 106236307 (+)
Alignment score:	1515	
E-value:	1.7e-260	
Alignment length:	**315**	
Percentage identity:	**97.14%**	

5.2.1.3 UOB-COL-1 clone 3 (serum CC005) identified as transcript of IGHG3 gene

Sequence fragment used for identification (T3 primer reading):

GGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGGGTGGAACTCAGGCGCTCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGG
ACTCTACTCCCTCAGCAGCGTGGTGACCGTGACCTCCAGCAACTTCGGCACCCAGACCTACACCTGCAACGTAGATCACAAGCCCAGCAACACCAAGGTGGACAAGACAGTTGAGC
GCAAATGTTGTGTCGAGTGCCCACCGTGCCCAGCACCACCTGTGGCAGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCAC
GTGCGTGGTGGTGGACGTGAGCCACGAAGACCCCGAGGTCCAGTTCAACTGGTACGTGGACGGCATGGAGGTGCATAATGCCAAGACAAAGCCACGGGAGGAGCAGTTCAACAGC
ACGTTCCGTGTGGTCAGCGTCCTCACCGTCNTGCACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGGCCTCCCAGCCCCCATCGAGAAAACCATCTCN
AAAACCAAAGGGNAGCCCCTANAACCACA

Fragment matched on Ensemble Genome Browser:

>chromosome **14:106235693:106236607:-1**

CTCCAGCTCAAGGCGGGACAAGAGCCCTAGAGTGGCCTGAGTCCAGGGACAGGCCCCAGCAGGGTGCTGACGCATCCACCTCCATCCC
AGATCCCCGTAACTCCCAATCTTCTCTCTGCAGAGCCCAAATCTTGTGACACACCTCCCCCGTGCCCAAGGTGCCCAGGTAAGCCAGCCC
AGGCCTCGCCCTCCAGCTCAAGGCAGGACAGGTGCCCTAGAGTGGCCTGCATCCAGGGACAGGTCCCAGTCGGGTGCTGACACATCTGC
CTCCATCTCTTCCTCAGCACCTGAACTCCTGGG**AGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGATACCCTTATGATTTC**
CCGGACCCCTGAGGTCACGTGCGTGGTGGTGGACGTGAGCCACGAAGACCCCGAGGTCCAGTTCAAGTGGTACGTGGACGGCG
TGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTTCCGTGTGGTCAGCGTCCTCACCGTCCTG

CACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTC
CAAAACCAAAGGTGGGACCCGCGGGGTATGAGGGCCACATGGACAGAGGCCAGCTTGACCCACCCTCTGCCCTGGGAGTGACCGCTGT
GCCAACCTCTGTCCCTACAGGACAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGAGGAGATGACCAAGAACCAGGTC
AGCCTGACCTGCCTGGTCAAAGGCTTCTACCCCAGCGACATCGCCGTGGAGTGGGAGAGCAGCGGGCAGCCGGAGAACAACTACAACA
CCACGCCTCCCATGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCA

*THIS STYLE: Location of selected alignment
*THIS STYLE: Location of other alignments
*THIS STYLE: Location of Exons

Alignment information:

Query location:	unnamed	**280** to **594** (-)
Database location:	14	106235993 to 106236307 (+)
Genomic location:	14	106235993 to 106236307 (+)
Alignment score:	1516	
E-value:	1.0e-260	
Alignment length:	**315**	
Percentage identity:	**96.83%**	

5.2.2 IGHG2 gene

Gene Description: **IGHG2**; Immunoglobulin heavy constant gamma 2 (G2m marker) located on chromosome 14.
Location: Chromosome 14: 106,109,389-106,111,127 reverse strand.
Transcripts: This gene has one transcript (splice variant)
Gene type: Known IG C gene.
Prediction method: Immunoglobulin (Ig) and T-cell receptor (TcR) annotation imported from the manually curated IG gene set from the Havana project. (It corresponds to Havana gene OTTHUMG00000152482).

5.2.2.1 UOB-COL-2 clone 1 (serum CC014) identified as a transcript of IGHG2 gene

Sequence fragment used for identification (T3 primer reading):

AAGTCCGCGGNGGCGGCCGCTCTAGACTGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGGGTGGNANTTAGGNGCTCTGACCAGCGGCGTGCACACCATTCCCGGCTGTCNTA
CAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGACCTCCAGCAACTTCGGCACCCAGACCTACACCTGCAACGTAGATCACAAGCCCAGCAACACCAAGGTGGACAAG
ACAGTTGAGCGCAAATGTTGTGTCAAGTGCCCACCGTGCCCAGCACCACCTGTGGCAGGACCGNCAGTCTTCCTCTTCCCCTCAAAACCCAAGGACACCCTCATGATCTCCCNGACC
CCNGAGGTCNCGTGCNTGNTGGTGAANAGNGAGANNACAAA

Fragment matched on Ensemble Genome Browser:

>chromosome **14:106110533:106111299:-1**

GGAGGGGGCTAAGGTGACGCAGGTGGCGCCAGCCAGGTGCACACCCAATGCCCGTGAGCCCAGACACTGGACCCTGCCTGGACCCTC
GCAGATAGACAAGAACCGAGGGGCCTCTGCGCCCTGGGCCCAGCTCTGTCCCACACCGCGGTCACATGGCACCACCTCTCTTGCAGCC
TCCACCAAGGGCCCATCGGTCTTCCCCCTGGCGCCCTGCTCCAGGAGCACCTCCGAGAGCACAGGGCCCTGGGCTGCCTGGTCAAGGA
CTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTC**AGGCGCTCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCC**
TCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAACTTCGGCACCCAGACCTACACCTGCAACGTAGATCA
CAAGCCCAGCAACACCAAGGTGGACAAGACAGTTGGTGAGAGGCCAGCTCAGGGAGGGAGGGTGTCTGCTGGAAGCCAGGCTCA
GCCCTCCTGCCTGGACGCACCCCGGCTGTGCAGCCCCAGCCCAGGGCAGCAAGGCAGGCCCCATCTGTCTCCTCACCCGGAGGCCTCT
GCCCGCCCCACTCATGCTCAGGGAGAGGGTCTTCTGGCTTTTTCCACCAGGCTCCAGGCAGGCACAGGCTGGGTGCCCCTACCCCAGG
CCCTTCACACACAGGGGCAGGTGCTTGGCTCAGACCTGCCAAAAGCCATATCCGGGAGGACCCTGCCCCTGACCT

***THIS STYLE:** Location of selected alignment
***THIS STYLE:** Location of other alignments
*THIS STYLE: Location of Exons

Alignment information:

Query location:	unnamed	**73** to **240** (-)
Database location:	14	106110833 to 106110999 (+)
Genomic location:	14	106110833 to 106110999 (+)
Alignment score:	803	
E-value:	5.9e-134	
Alignment length:	**168**	
Percentage identity:	**97.62%**	

5.2.2.2 UOB-COL-2 clone 2 (serum CC014) identified as transcript of IGHG2 gene

Sequence fragment used for identification (T3 primer reading):

GCAGGAATTCGGCACGAGGGTGGANCTCATGCGCTCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGACCT
CCAGCAACTTCGGCACCCAGACCTACACCTGCAACGTAGATCACAAGCCCAGCAACACCAAGGTGGACAAGACAGTTGAGCGCAAATGTTGTGTCGAGTGCCCACCGTGCCCAGCA
CCACCTGTGGCAGGACCGTCAGTCTTCCTCTTCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACGTGCGTGGTGGTGGACGTGAGCCACNAAGACCCC
GAGGTCCAGTTCAACTGGTACGTGGACGGCATGGAGGTGCATAATGNCAAGACAAAGCCACGGGAGGAGCAGTTCAACAGCACGTTCCGTGTGGTCAGCGTCCTCACCGTCGTGCA
CCATGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGGCCTCCCAGCCCCCATCGAGAAAANNATCTCCAAAACCAAAGGGCNNNCCCGAGAANCACAGGTG
TACACCCTGNCCCNATCCCGGGAGGANATNACNAGGAACCATGTCANNNTGANNTGCNTGGTCNANGGCNTCTACCCCAGTNGACNTCCTCNTGGANNGNTACAGCAATGGGAAGT
TTGG

Fragment matched on Ensemble Genome Browser:

>chromosome **14: 106109660:106110586:-1**

TGCTTGGCTCAGACCTGCCAAAAGCCATATCCGGGAGGACCCTGCCCCTGACCTAAGCCGACCCCAAAGGCCAAACTGTCCACTCCCT
CAGCTCGGACACCTTCTCTCCTCCCAGATCCGAGTAACTCCCAATCTTCTCTCTGCAG**AGCGCAAATGTTGTGTCGAGTGCCCACCG**
TGCCCAGGTAAGCCAGCCCAGGCCTCGCCCTCCAGCTCAAGGCGGGACAGGTGCCCTAGAGTAGCCTGCATCCAGGGACAGACCCCA
GCTGGGTGCTGACACGTCCACCTCCATCTCTTCCTCAG**CACCACCTGTGGCAGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCA**
AGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACGTGCGTGGTGGTGGACGTGAGCCACGAAGACCCCGAGGTCCAGTT
CAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCACGGGAGGAGCAGTTCAACAGCACGTTCCGTGTG
GTCAGCGTCCTCACCGTCGTGCACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGGCCTCCCAG
CCCCCATCGAGAAAACCATCTCCAAAACCAAAGGTGGGACCCGCGGGGTATGAGGGCCACATGGACAGAGGCCGGCTCGGCCCACC
CTCTGCCCTGGGAGTGACCGCTGTGCCAACCTCTGTCCCTACAG**GGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCC**
GGGAGGAGATGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTACCCCAGCGACATCTCCGTGGAGTGGGAGA
GCAATGGGCAGCCGGAGAACAACTACAAGACCACACCTCCCATGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGC

***THIS STYLE:** Location of selected alignment
***THIS STYLE:** Location of other alignments
*THIS STYLE: Location of Exons

Alignment information:

Query location:	unnamed	**232** to **558** (-)
Database location:	14	106109960 to 106110286 (+)
Genomic location:	14	106109960 to 106110286 (+)
Alignment score:	1618	
E-value:	9.2e-279	
Alignment length:	**327**	
Percentage identity:	**98.17%**	

5.2.3 CYB5R3 gene

Gene Description:	**CYB5R3**; Cytochrome b5 reductase 3 located on Chromosome 22
Location:	Chromosome 22: 43,013,846-43,045,574 reverse strand.
Transcripts:	This gene has nine transcripts (splice variants)
Gene type:	Known protein coding.
Prediction method:	Annotation for this gene includes both automatic one from Ensembl and a Havana manual curation. It corresponds to Havana gene OTTHUMG00000150745 (version 5).

5.2.3.1 UOB-COL-4 clone 1 (serum CC005) identified as transcript of CYB5R3 gene

Sequence fragment used for identification (T3 primer reading):

CGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGGCATCCCTGATGGCAGAACTCCCACAACCACATGTATTTATTCCTCTGCTCCTAAACC
GTCCCCTCCTTCCCTCACCCCCAGCACAGGGGGATTCTGAGCAGTGCCTCTTGTCTGAGGGACATATCAGTGACCTCGACGTTGCCTTTAGACTACAGTTGTGTTAGCCTCTTGCGTAT
TGGCTTTTTCAGAGTCATTTATGAGCAAAAAAAAAAAAAAAAA

Fragment matched on Ensemble Genome Browser:

>chromosome **22:43014540:43015353:-1**

GTCACCCCCACTCCCGCCTCAGGGCCAGGCCCAGGCCTCACCACCTGACGCTGCATGAGACATTGACACCAGAAAGCCCTCTTGGGG
GCACTGCTCCCTACCCCAGGGCCCTGGCCAGCCGGGAGCTTGGCTCTCCTCTGGCTAGAGTGGGAAGAGGGGGCTGGCCATGGGGCC
CTCCCAGAACCTCAGCATTTCCTTCCAGCCCATCCAAACACTGAGGCAGCCTTGGGGAACCCCGAGCTGGGGGGTTGGCAGCCCACTG
CACCGCCTCAGGGTTTTGGGGTCCTGGGCTGGGGCCAC**CATCCCTGATGGCAGAACTCCCACAACCACATGTATTTATTCCTCTGT**
CCTAAACCGTCCCCTCCTTCCCTCACCCCCAGCACAGGGGGATTCTGAGCAGTGCCTCTTGTCTGAGGGACATATCAGTGACC
TCGACGTTGCCTTTAGACTACAGTTGTGTTAGCCTCTTGCGTATTGGCTTTTTCAGAGTCATTTATGAGCAGAAAAAAAAAAAG
TAAAACTTTGCTAATATTAACCCTTCTCTAGCTCCTCGAGGGTCTGTGACCTGCAACACAAGGGGTGGGGTCAGGAAAGGGCTGGGGA
AGACCTAGCATTTTTTTTTTCTTTTTTTTTTTTTTTTTTGAGACGGAGTCTCCCTTTGTCACCCAGGCTGGAGTGGCATGATCTCAGCTCACT
ACAACCTCCACCTCTCGGGTTCAAGCGATTCTCCTGCCTCAGCCTCCCGAGTAGCTGGGACTAAAAGTGCCCACCACCACACCCAGCT
AGTTTTTGTATTTTTTTTTTTTTTTTTTGAG

***THIS STYLE:** Location of selected alignment
***THIS STYLE:** Location of other alignments

*THIS STYLE: Location of Exons

Alignment information:

Query location:	unnamed	**61** to **275** (-)
Database location:	22	43014840 to 43015053 (+)
Genomic location:	22	43014840 to 43015053 (+)
Alignment score:	1043	
E-value:	1.1e-176	
Alignment length:	**215**	
Percentage identity:	**99.07%**	

5.2.3.2 UOB-COL-4 clone 3 new (serum CC005) identified as transcript of CYB5R3 gene

Sequence fragment used for identification (T3 primer reading):

GGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGNCTGCAGGAATTCGGCACGAGGCATCCCTGATGGCANAACTCCCACAACCACATGTATTTATTCCTCTGCTCCTAAACCGTCCC
CTCCTNCCCTCACCCCCAGCACAGGGGGATTCTGAGCAGTGCCTCTTGTCTGAGGGACATATCAGTGACCTCGACGTTGCCTTTAGACTACAGTTGTGTTAGCCTCTTGCGTATTGGCT
TTTTCAGAGTCATTTATGAGCAAAAAAAAAAAAAAAA

Fragment matched on Ensemble Genome Browser:

>chromosome **22:43014540:43015353:-1**
GTCACCCCCACTCCCGCCTCAGGGCCAGGCCCAGGCCTCACCACCTGACGCTGCATGAGACATTGACACCAGAAAGCCCTCTTGGGG
GCACTGCTCCCTACCCCAGGGCCCTGGCCAGCCGGGAGCTTGGCTCTCCTCTGGCTAGAGTGGGAAGAGGGGGCTGGCCATGGGGCC
CTCCCAGAACCTCAGCATTTCCTTCCAGCCCATCCAAACACTGAGGCAGCCTTGGGGAACCCCGAGCTGGGGGGTTGGCAGCCCACTG
CACCGCCTCAGGGTTTTGGGGTCCTGGGCTGGGGCCAC**CATCCCTGATGGCAGAACTCCCACAACCACATGTATTTATTCCTCTGT**
CCTAAACCGTCCCCTCCTTCCCTCACCCCCAGCACAGGGGGATTCTGAGCAGTGCCTCTTGTCTGAGGGACATATCAGTGACC
TCGACGTTGCCTTTAGACTACAGTTGTGTTAGCCTCTTGCGTATTGGCTTTTTCAGAGTCATTTATGAGCAGAAAAAAAAAAAAG
TAAAACTTTGCTAATATTAACCCTTCTCTAGCTCCTCGAGGGTCTGTGACCTGCAACACAAGGGGTGGGGTCAGGAAAGGGCTGGGGA
AGACCTAGCATTTTTTTTTTCTTTTTTTTTTTTTTTTTGAGACGGAGTCTCCCTTTGTCACCCAGGCTGGAGTGGCATGATCTCAGCTCACT
ACAACCTCCACCTCTCGGGTTCAAGCGATTCTCCTGCCTCAGCCTCCCGAGTAGCTGGGACTAAAAGTGCCCACCACCACACCCAGCT
AGTTTTTGTATTTTTTTTTTTTTTTTTTGAG

***THIS STYLE:** Location of selected alignment
***THIS STYLE:** Location of other alignments
*THIS STYLE: Location of Exons

Alignment information:

Query location:	unnamed	**56** to **270** (-)
Database location:	22	43014840 to 43015053 (+)
Genomic location:	22	43014840 to 43015053 (+)
Alignment score:	1033	
E-value:	16.9e-175	
Alignment length:	**215**	
Percentage identity:	**98.14%**	

5.2.4 RPL37A gene

Gene Description: Ribosomal protein L37a located on Chromosome 2.
Location: Chromosome 2: 217,362,912-217,443,903 forward strand.
Transcripts: This gene has twelve transcripts (splice variants).
Gene type: Known protein coding.
Prediction method: Annotation for this gene includes both automatic one from Ensembl and a Havana manual curation. It corresponds to Havana gene OTTHUMG00000133052 (version 4).

5.2.4.1 UOB-COL-11 clone 2 (serum CC005) identified as transcript of RPL37A gene

Sequence fragment used for identification (T3 primer reading):

CGCGGTGGCGGCCGCTCTAGAAACTAGTTGGATCCCCCGGGGCTGCAGGAATTCGGCACGAGGCTAGGTCGCGGCGACATGGCCAAACGTACCAAGAAAGTCGGGATCGTCGGTAA
ATACGGGACCCGCTATGGGGCCTCCCTCCGGAAAATGGTGAAGAAAATTGAAATCAGCCAGCACGCCAAGTACACTTGCTCTTTCTGTGGCAAAACCAAGATGAAGAGACGAGCTG
TGGGGATCTGGCACTGTGGTTCCTGCATGAAGACAGTGGCTGGCGGTGCCTGGACGTACAATACCACTTCCGCTGTCACGGTAAAGTCCGCCATCAGAAGACTGAAGGAGTTGAAAG
ACCAGTAGACGCTCCTCTACTCTTTGAGACATCACTGGCCTATAATAAATGGGTTAATTTATGTAAAAAAAAAAAAAAAA

Fragment matched on Ensemble Genome Browser:

>chromosome **2:217363693:217364421:1**
CCGTGTTCTCTCCTGTCTCCATGCCTTTGCAGGAGACACCATTGTCGGAAGCTCCCCAAGGCGGAGGGGCGGGGGCGCCTTGGCTGGG
CCTGGCGCGCTCCAGCCGGGTTAACGCCGGGCCTTCGGAGCGCGCGGCCAGCCCTGGGCACTGGTTTCGTTGGGTTGAATTTAGGGAA
AACTAGGTCATATGTAATTCACATGTCGGTCACACGTCAGTGAGGTGGAGGAACGGTGTGTGGAGGCTCCAGGGCCTGCCTGGGTTCC
AGGTCTATCACTGGTTTCTCCCTTCACTCTAAACAG**GCCAAACGTACCAAGAAAGTCGGGATCGTCGGTAAATACGGGACCCGCT**
ATGGGGCCTCCCTCCGGAAAATGGTGAAGAAAATTGAAATCAGCCAGCACGCCAAGTACACTTGCTCTTTCTGTGGCAAAGTA
AGTAAGGCAAAGTCTCTGGTGAGAGGAGAGGGAGGGCAGGTTTCTTACCCAAGTGAGGCCTGACTTCAAGGTATTTTATAAGCCGTG
TGCTGGTGGGCAGTTGGAATTACTCATACCGTTGATTATGAGTTTTAAGATAAAAGTGTTGATGGTAACTTCAGATTTTGTGAGACGTT
TTTCATTTAAAGAAAACCGCTTAAACGTTAATGGGTAAAATAATCATTTGACAGAGTGCCCCCAGCCTAAGCCAAACCTGCTTTGTGG
GAAATGATTCCATCAGTTTTGTCTACTGATGTT

***THIS STYLE:** Location of selected alignment
***THIS STYLE:** Location of other alignments
*THIS STYLE: Location of Exons

Alignment information:

Query location: unnamed **82** to **210** (+)
Database location: 2 217363993 to 217364121 (+)
Genomic location: 2 217363993 to 217364121 (+)
Alignment score: 649
E-value: 1.4e-106
Alignment length: **129**
Percentage identity: **100.00%**

5.2.4.2 UOB-COL-11 clone 4 (serum CC005) identified as transcript of RPL37A gene

Sequence fragment used for identification (T3 primer reading):

CGGNGGGCGGCCGCTCTAGAACTAGTTGGNATCCCCCGGGNCTGCAGGTAATTCGGCACGAGGCTACNGTTTNCGGCGNACATGGCCAAACGTACCAAGAAAGTCGGGATCNTNTT
GTAAATACGGGACCCGCTATGGGGCCTCCCATCCG

Fragment matched on Ensemble Genome Browser:

>chromosome **2:217363724:217364357:1**
GGAGACACCATTGTCGGAAGCTCCCCAAGGCGGAGGGGCGGGGGCGCCTTGGCTGGGCCTGGCGCGCTCCAGCCGGGTTAACGCCGG
GCCTTCGGAGCGCGCGGCCAGCCCTGGGCACTGGTTTCGTTGGGTTGAATTTAGGGAAAACTAGGTCATATGTAATTCACATGTCGGT
CACACGTCAGTGAGGTGGAGGAACGGTGTGTGGAGGCTCCAGGGCCTGCCTGGGTTCCAGGTCTATCACTGGTTTCTCCCTTCACTCT
AAACA**GGCCAAACGTACCAAGAAAGTCGGGATCGT**CG**GTAAATACGGGACCCGCTATGGGGCCTCCCTCCG**GAAAATGGTGAA
GAAAATTGAAATCAGCCAGCACGCCAAGTACACTTGCTCTTTCTGTGGCAAAGTAAGTAAGGCAAAGTCTCTGGTGAGAGGAGAGGG
AGGGCAGGTTTCTTACCCAAGTGAGGCCTGACTTCAAGGTATTTTATAAGCCGTGTGCTGGTGGGCAGTTGGAATTACTCATACCGTT
GATTATGAGTTTTAAGATAAAAGTGTTGATGGTAACTTCAGATTTTGTGAGACGTTTTTCATTTAAAGAAAACCGCTTAAACGTTAATG
GGTAAAATAATCATTTGACAGAGT

***THIS STYLE:** Location of selected alignment
***THIS STYLE:** Location of other alignments
*THIS STYLE: Location of Exons

Alignment information:

Query location:	unnamed	**117** to **151** (+)
Database location:	2	217364024 to 217364057 (+)
Genomic location:	2	217364024 to 217364057 (+)
Alignment score:	150	
E-value:	5.2e-18	
Alignment length:	**35**	
Percentage identity:	**97.14%**	

5.2.5 SLC34A2 gene

Gene Description: **SLC34A2**; Solute carrier family 34 (type II sodium/phosphate contrasporter), member 2.
Location: Chromosome 4: 25,656,923-25,680,370 forward strand.
Transcripts: This gene has six transcripts (splice variants).
Gene type: Known protein coding.
Prediction method: Annotation for this gene includes both automatic one from Ensembl and a Havana manual curation. It corresponds to Havana gene OTTHUMG00000097757 (version 3).

5.2.5.1 UOB-COL-5 clone 1 (serum CC014) identified as transcript of SLC34A2 gene

Sequence fragment used for identification (T3 primer reading):

CTCNCGCGGTGGCGGCCGCCTCTAGAACTAGTGGATCCCCCGGGGCTGCAGGAATTCGGCACGAGGGCAAGATGGGTCACCAGCAGCTGTACTGGAGCCACCCGCNAAAATTCGGC
CAGGGTTCTCGCTCTTGTCGTGTCTGTTCAAACCGGCACGGTCTGATCCGGAAATATGGCCTCAATATGTGCCGCCAGTGTTTCCGTCAGTACGCNAAGGATATCGGTTTCATTAAGT
TGGACTAAATGCTCTTCCTTCAGAGGATTATCCGGGGCATCTACTCAATGAAAAACCATGATAATTCTTTGTATATAAAATAAACATTTGAAAAAAAAAANAAAAA

Fragment matched on Ensemble Genome Browser:

>chromosome **3:196263098:196263974**

GTTTCATAAACATAAACAAGGATGCCATGACCCTGGGAAATAACATTTTTTGGAAAATAATTTTTAAACTTTTGAAATTATTTTTTTCT
TTTTTTGAGACAGAGTCTCGCTTTGTAGCCTAGGCTGGTGTGCAGTGGCGCGATCTTGGCTCGCTGCAACCTCTGCCTCCTGGATTCAA
GCGATTGTCCTGCCTCAGCCGCCTGAGTAGCTGGGATTACAGGCGTGAGCCACCACGCCCGGGGAATTTTTGTATGTTTAGAAGAGAC
GGGGTTCCCCTTTTACCTCGTTGCACTCCTGAGA**GCAAGATGGGTCACCAGCAGCTGTACTGGAGCCACGCGCGAAAATTCGGCC**
AGGGTTCTCGCTCTTGTCGCGTCTGCTCAAACCGGCACGGTCTGATCCGGAAATATGGCCTCAATATGCGCCGCCAGTGTTTC
CGTCAGTACGCGAAGGATGTCGGTTTCATTAAGTTGGACTAAATGATCTTCCTTCAAAGGGTTATCCAAGGCATCTACTCAAT
GAAAAACCATGATCATTCTTTGTACATAAAATAAACATTTGAAAAACCCAAAAAAAAAAAAAAAAAAAAAGAGATGGGGTTTCAC
CATGTTGGCCAGGCTGGTTTTGAACTCCTGGTCTCAAGTTATCTGCCCGCCTCGTCCTCCCAAAGTGCTGGGATTACAGGCATGAGCCA
ATGTGCTCAGCCCTGGAAATTTAAAATAGATTGATTGTAAAGAAATCTACTTGGGCAAAAAAATTTTAAATTTGAATACTGAAATAAT
AATGGTTCCTTAGAGCCATAAAGTGTTTATTAACAAGAAATACAGTTGATAAAAGATATGTATGATTGCCCAACTTACATAGAAAAGA
AAAAAATGGAA

***THIS STYLE:** Location of selected alignment
***THIS STYLE:** Location of other alignments
*THIS STYLE: Location of Exons

Alignment information:

Query location:	unnamed	**67** to **340** (-)
Database location:	3	196263398 to 196263676 (+)
Genomic location:	3	196263398 to 196263676 (+)
Alignment score:	1234	
E-value:	1.2e-210	
Alignment length:	**277**	
Percentage identity:	**93.50%**	

CHAPTER 6 Results: Identification of known antigens expressed in SW480 cell line

6.1. mRNA extraction from SW480 cell line and cDNA synthesis

Total mRNA was extracted from the SW480 cell line and used to synthesize cDNA fragments that are suitable for a PCR. The samples were stored at small aliquoted in the -20 lab freezer.

6.2 PCR optimization and antigen identification

The newly synthesized cDNA from the mRNAs isolated from SW480 cell line was used to set a PCR reaction in the search for the expression of known antigens. Two individual cDNA samples were used as templates for the PCR reactions (cDNA 1 and cDNA 2) (Section 6.1).

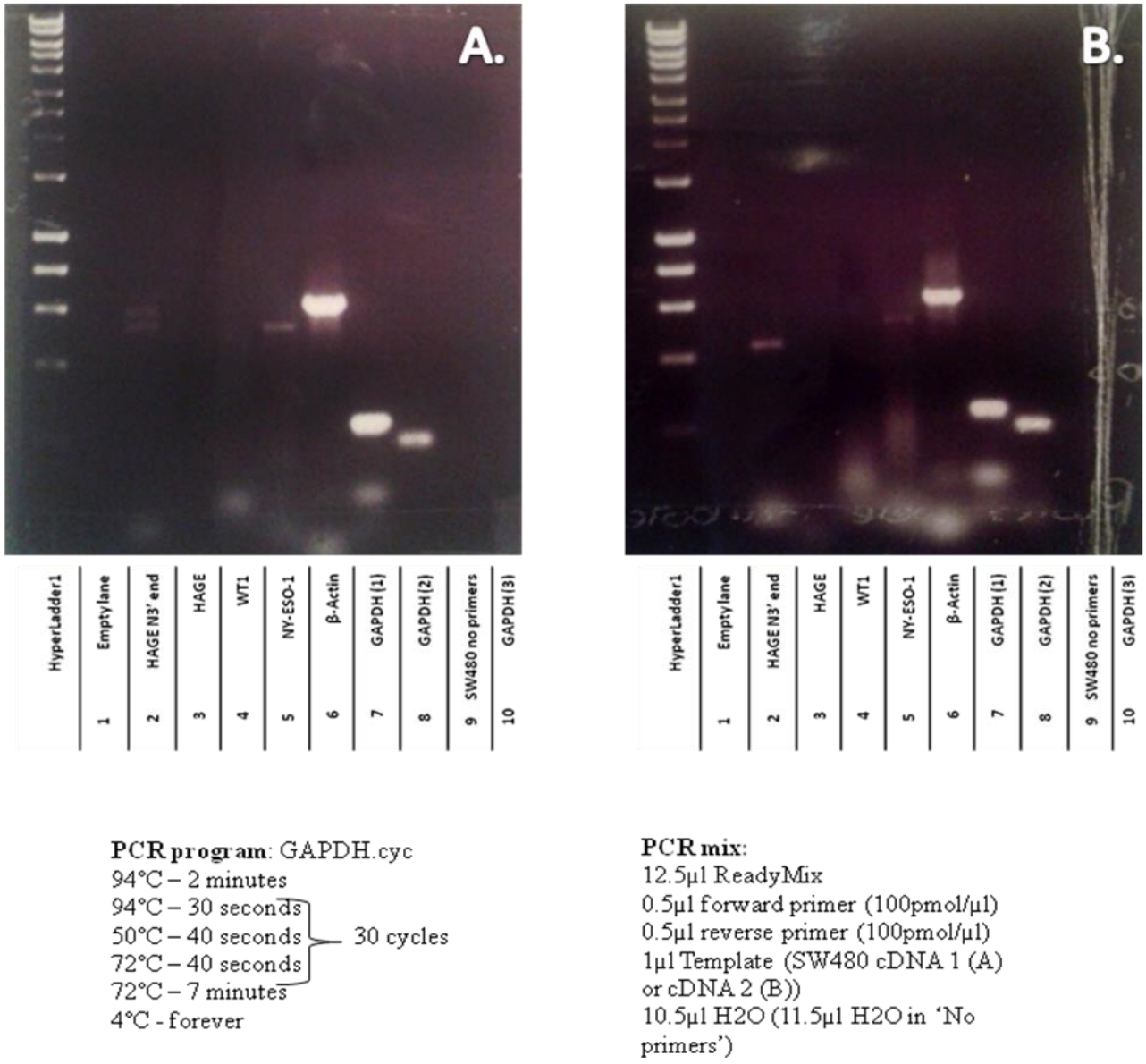


Figure 6.1 Expression of known antigens in SW480 cell line. (**A**) cDNA 1 used as a template and (**B**) cDNA 2 used as a template; β-Acting and GAPDH were the housekeeping genes chosen to serve as positive controls in both occasions. Lane 9 on (**A**) and (**B**) is a negative control, containing only the template and no primers.

It appeared that the PCR conditions used for this reaction were suitable to amplify β-Acting and GAPDH in both occasions. Also NY-ESO-1 expression was detected in both cDNA samples from SW480. However, HAGE and WT1 primer pairs did not amplify a product in these reactions. Interestingly, cDNA 1

had produced a double band for HAGE near 3' end (**Figure 6.1 A**) in comparisosn to a single band for the same target sequence in cDNA 2 (**Figure 6.1 B**).

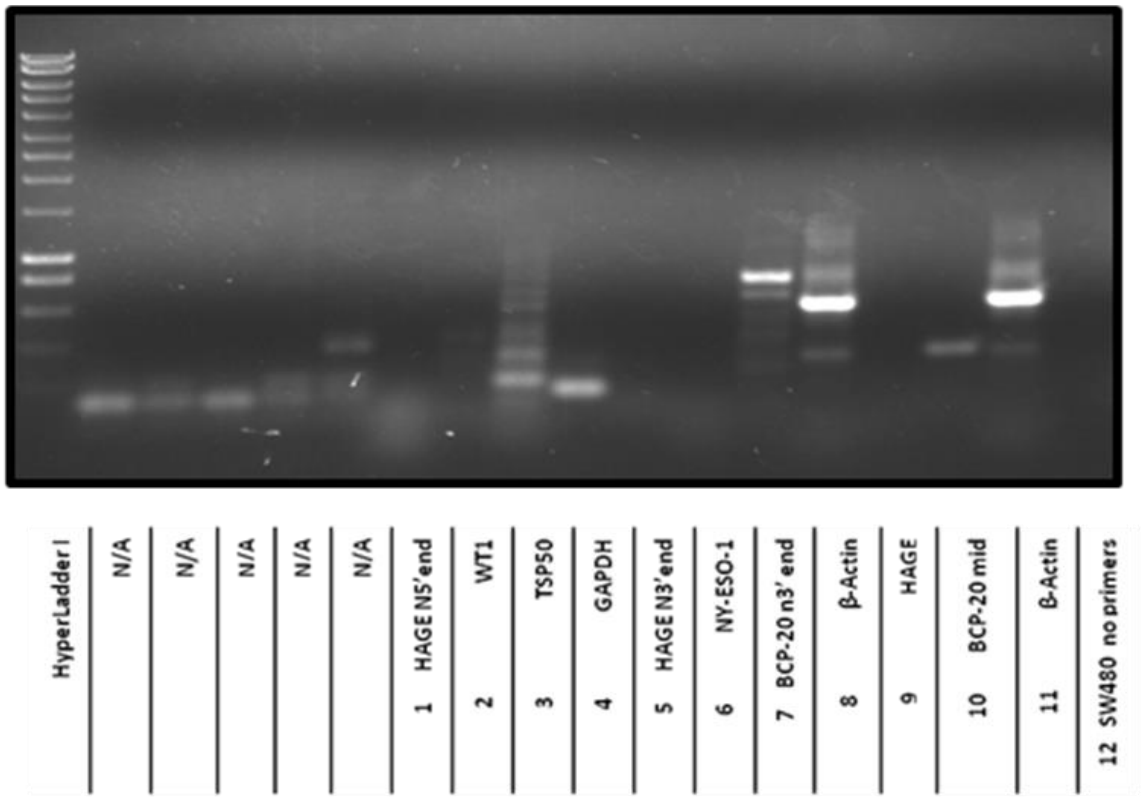
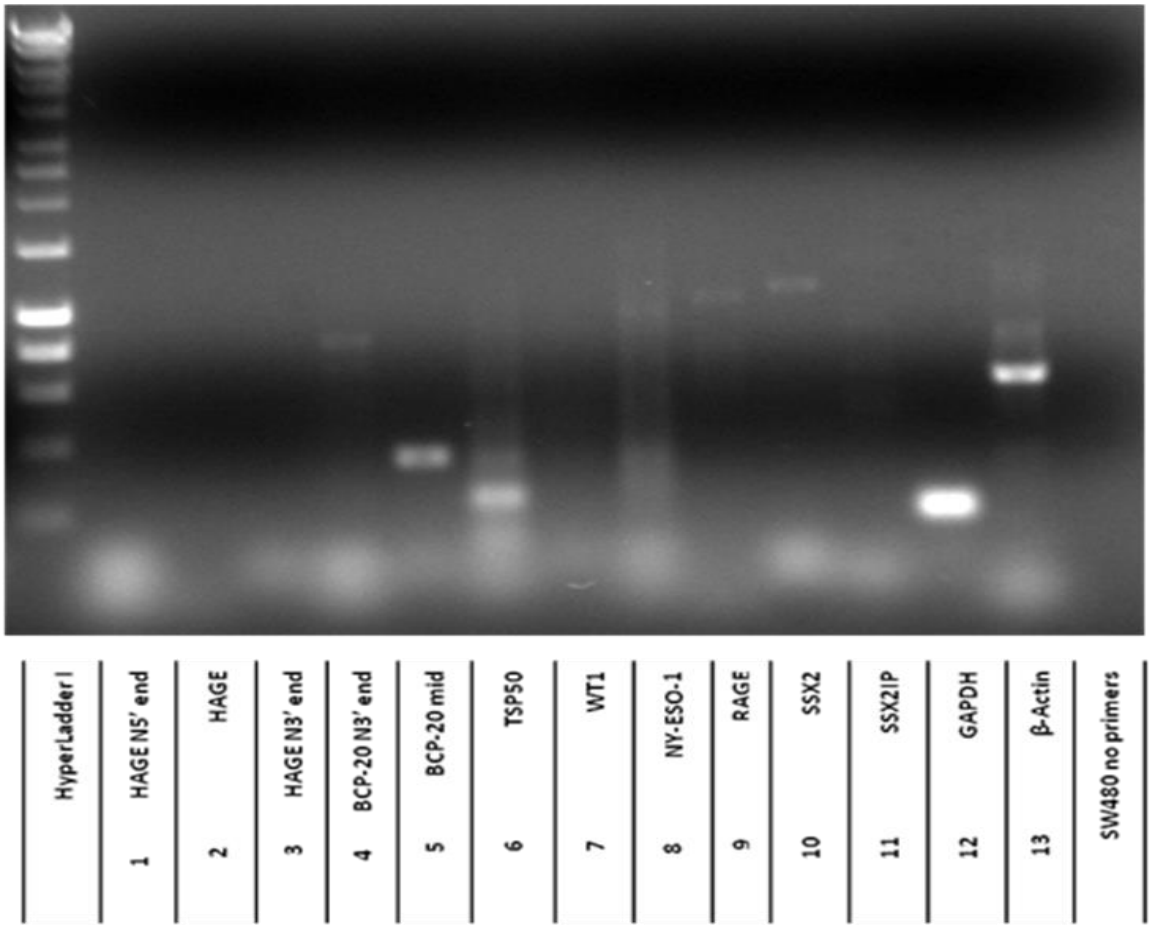


Figure 6.2 Expression of known antigens in SW480 cell line. PCR conditions appeared favourable for the detection of WT1 expression, although the amount of the PCR product appeared low. Further optimisation should increase the concentration of DNA produced, knowing that WT1 is expressed in SW480 cell line. No product amplified by the NY-ESO-1 primer pair (conditions unfavourable). Also, TSP50 had a smear on the gel and will need further optimisation of the conditions.

For the following PCR reaction (**Figure 6.3**), the conditions were altered to resemble the ones used in **Figure 6.1** as the bands produced there appeared well-defined.



PCR program: GAPDH.cyc
94°C – 2 minutes
94°C – 30 seconds
50°C – 40 seconds } 40 cycles
72°C – 40 seconds
72°C – 7 minutes
4°C - forever

PCR mix:
12.5µl ReadyMix
0.5µl forward primer (100pmol/µl)
0.5µl reverse primer (100pmol/µl)
1µl Template (SW480 cDNA)
10.5µl H2O (11.5µl H2O in 'No primers')

Figure 6.3 Expression of known antigens in SW480 cell line. Some of the bands observed in Figure 6.3 were very clear (BCP-20 mid, BCP-20 N5' end, RAGE and SSX2). However, HAGE N5' end primer pair did not produce a band regardless of the PCR conditions being similar to the ones in **Figure. 6.1.** WT1 primer pair did not produce a band as the annealing t˚ was reduced by 2˚C in comparison to **Figure 6.2.**

Table 6.1 Expression of known antigens in SW480 cell line as identified by PCR.

Antigen	Primer sequence 5'-3'	Temperature of Melting (T_m) ˚C	Best annealing temperature for product (T_a)	Estimated product length (bp)	Product length as seen (average bp)
β-Actin	F: GGCATCGTGATGGACTCCG R: GCTGGAAGGTGGACAGCGA	69.2 69.0	50˚C - 60˚C	Not known	620
GAPDH	F: ACCCACTCCTCCACCTTTG R: CTCTTGTGCTCTTGCTGGG	64.0 63.8	50˚C - 60˚C	Not known	220
TSP50	F: TGACGGCATGTGGCCTCAGTT R: TCAGAGGGCAGCAAGGAGGCT	72.3 71.7	50˚C and 52˚C	401	300
HAGE (near 3' end)	F: CTGTACTAGTGGGGTAGAGAATTCA R: CACAGAAACGAACATTTATTAACAG	61.3 60.4	50˚C	351	450
HAGE (near 5' end)	F: TTTGTTGGCGCGGTAATCGGT R: CATGGCACTTGTGGCAGTGGA	72.1 71.8	50˚C and 52˚C no product	351	Not seen
HAGE	F: CCTTTCAATGTTATCCTGAG R: TATTCTTCAGATTGACGAAG	56.2 54.4	50˚C and 52˚C no product	Not known	Not seen
BCP-20 (near 3' end)	F: ATGAGTCACTCGACGAGGAGC R: TTTAAAGCCACCTGGAGCCT	65.9 64.7	52˚C	451	600-800
BCP-20 (middle)	F: TCATTCATCAGCAGCTTGAGC R: GAACGTGGCATTTGATGTTGA	65.6 65.8	50˚C	301	360
WT1	F: GAGAGCGATAACCACACAAC R: GATGACCAAACTCCAGCTGG	59.8 64.7	52˚C	Not known	420
SSX2	F: AAAATCAGAGTCAGACTGCTCCCGGTG R: GTACATGCTGACCAGGAAACAGAGTGA	73.5 70.0	50˚C	Not known	1100
SSX2IP	F: TGAATGAGCTGCTTGTGCTT R: GCTGATGCAAATTCCTGTTCT	63.7 63.1	50˚C no product	Not known	Not seen
NY-ESO-1	F: CCCCACCGCTTCCCGTG R: CTGGCCACTCGTGCTGGGA	72.6 72.4	50˚C	Not known	450
RAGE	F: GTGTCTCCTTCGTCTCTACTA R: GGTGTGCCGATGACATCG	55.1 66.7	50˚C	Not known	1000

CHAPTER 7 General Discussion

SEREX has been used to identify novel antigens such as NY-ESO-1 (Chen *et al*, 1997), CT antigens such as PASD1 (Guinn *et al*, 2005) and known tumour antigens such as SSX2IP (Guinn *et al*, 2005). We used the SEREX technique to identify which antigens are being recognised by Duke's B colon cancer sera. We focussed on patients who had reactive disease to see what they react to and how that differs to patients with non-reactive Duke's B colon cancer. We choose to use SEREX as it can identify IgG responses and has already been shown to identify antigens also recognised by $CD8^+$ T cells. It is these cells which are capable of specifically killing tumour cells based on the MHC class I presentation of tumour specific antigens.

Most SEREX antigens which have been identified have been transcription factors. Nuclear antigens with poor immunogenicity. Those antigens which naturally engage B cell – antibody mediated responses as the tumour cell are likely to have already caused the lysis of their host cell. Imunogenic antigens which are presented on MHC class I, antigens created by mutation or overexpression will have led to the elimination of tumour cells during the early stages of tumour development.

However it is thought that the antigens identified by SEREX are poorly immunogenic and played no part in the early elimination of the tumour cell (and hence still exist). In 2004, Robert Schreiber (Schreiber, 2004) described the three phases of immune surveillance and evasion (**Figure 7.1**). In. the early stages of tumour development the immune system recognizes and eliminates tumour cells very efficiently and prevents solid tumor from forming. This is proven to occur as patients who are immune compromised (those with AIDS, SCID or post-transplant) as these patients have enhanced rates of tumour development, suggesting that in healthy individuals when the immune system is

intact it can destroy tumours before they are detectable. Eventually, a process of selection gives rise to a cancer cell that can evade immune surveillance by virtue of down regulated tumour antigen, MHC, the absence of co-stimulatory molecules which can induce anergy. At this mid-stage the tumour may exist as a heterogeneous ball of cells, some of which have more acquired more or different mutations. Genetic instability is one of the hallmarks of cancer (**Figure 7.2**) and as the tumour evolves it acquires mutations that lead to grow and develop while avoiding immune destruction. In this phase, there is no net growth of tumor. Cells are still being eliminated and replaced in the tumor. This can continue for years. Then, some tumor cells escape, micrometasis in the case of colon cancer and invade the deeper layer of tissues in the bowel, leading to a detectable cancer.

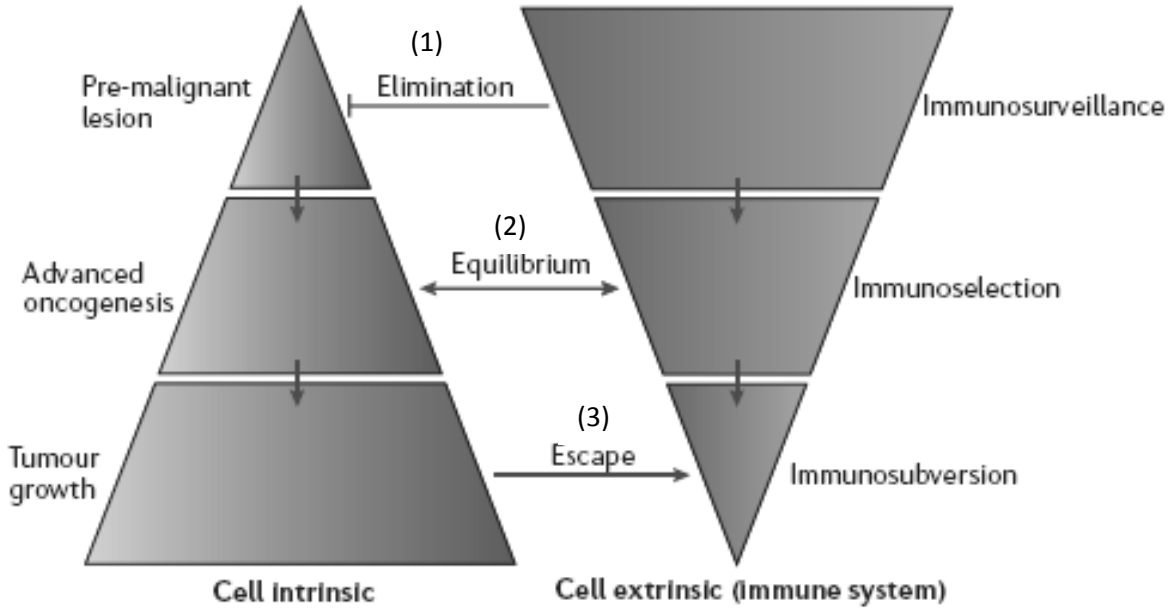


Figure 7.1 The three Es hypothesis. Figure based on Schreiber, 2004 theory, image taken from Zitvogel *et al*, 2006. The three phases of tumour development – (1) elimination where the tumour is detected and destroyed by the immune system (2) equilibrium – where as many tumour cells are killed by the immune system as are being born as daughter cells (3) escape where the tumour cells exceed the ability of the immune system to kill them and the tumour becomes detectable.

Figure 7.2 The seventh hallmark of cancer. Following the six hallmarks of cancer self-sufficiency in growth signals, insensitivity to growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and evading apoptosis described by Hannahan and Weinberg in 2000, a number of authors have noted the seventh hallmark of cancer appears to be lack of immune rejection (Dunn *et al*, 2004; Zitvogel *et al*, 2006; Curiel, 2007). Based on the three E's theory of Schreiber, 2004 this would change the tumour from one in equilibrium to one that has escaped immune surveillance and can grow unchecked. Usually, the point at which tumours are clinically detectable and impacting on human health.

By the time patients have been diagnosed it is often late in the cancer development then it has impacted on health and wellbeing. Cancer tends to kill due to metastases when the cancer cells fill up spaces required for normal healthy functions. Many treatments work well at first and then tumours become resistant to the treatment. Colon cancer is often treated by surgery and chemotherapy. However micrometastases can lead to subsequent relapse and death. It has been hoped that immunotherapy can help remove these micrometastases and delay or prevent relapse. However in 2004 Rosenberg *et al* described the very poor results of immunotherapy trials at their centre which they felt reflected the general state-of-the-art in other Institutions. The paper described the 2.6% success of all immunotherapy clinical trials when assessing outcomes for patients undergoing immunotherapy clinical trials (n=440) in the Surgery Branch of the National Cancer Institute from 1995-2004. Objective responses were determined based on the Response Evaluation Criteria in Solid Tumors (RECIST) whereby an objective remission was scored as at least a 50% decrease in the sum of the products of the perpendicular lesions without a 35% growth of any lesions and no appearance of new lesions.

The big problems that immunotherapy faces are:-

- Anti-tumour immunity is autoimmunity.
- The generation of significant anti-tumour immunity requires breaking self tolerance.

Rosenberg's publication led to the need for a rethink of how we develop immunotherapy and a decision was made to focus efforts on specific antigen targets to focus funding and effort. To this end in 2010 a list of top 20 antigens which should be targeted by immunotherapy and required/deserved further investigation was published.

To prioritize the myriad of identified antigens, Cheever *et al.* (2009) devised a list of criteria to define the "ideal" target antigen with the order representing the weight of each characteristics. These include inducing clinical effects, being immunogenic, playing a critical role in cell differentiation and proliferation of the malignant cells or proteins which characterize the malignant cell, expression restricted to malignant cells, expression in malignant stem cells, high number of patients bearing antigen-positive cells, includes multiple antigenic epitopes and expressed on the cell surface. However there is no antigen that fulfills all of the criteria some antigens seem to be more suited for use in clinical vaccination than others.

In our study, based on an idea by Professor Gerald O' Sullivan, Cork Cancer Research Centre, University College Cork, we wished to determine what antigens were recognised by the immune cells in Duke's B reactive disease. This small cohort of patients has relatively early colon cancer, at the stage at which it is most often diagnosed. On conventional treatment these patients respond significantly better as detailed by Murphy *et al*, 2000. The group noticed that a proportion of patients (7/12) with Duke's B reactive disease had clearance of their micrometastases after surgery. In contrast 9/24 patients without detectable micrometastases before surgery had micrometastases after surgery, suggesting in the former group a role of the immune system in tumour clearance after surgery. The presence of micrometasases after surgery has been shown to be an indicator of residual disease and poor prognosis (O'Sullivan *et al*, 1997) and indeed the clearance of micrometastases post-surgery has been suggested to be a good prognostic indicator (Murphy *et al*, 2000). The importance of an immune response and primary colon cancer with regards to patient survival has already been established particularly through the detection of tumour infiltrating lymphocytes (Jass, 1986; Graham & Appelman, 1990; Ropponen *et al*, 1997), a Crohn's-like lymphoid reaction and extramural vein invasion (Harrison *et al*, 1994) played key roles in the refinement of Jass

classification system (Jass *et al*, 1987). Murphy *et al*, 2000 showed that histopathological identification of the inflammatory reaction in the primary tumour can provide a reproducible prognostic indicator of a 10-year survival advantage to these patients.

Antibody responses from B cells, require CD4 help, and therefore should also stimulate CD8 responses. CD8 responses are believed to be essential for effective anti-tumour responses. If we can stimulate these and break tolerance then we can cause tumour lysis. These T cells have the potential to remove micrometastases which are a major issue in colon cancer patients with non-reactive Duke's B colon cancer and are believed to be one of the most important indicators of their survival prospects.

In our study we identified two antigens involved in inflammatory responses. UOB-COL-1-c1 was found to encode the immunoglobulin heavy constant gamma 3 (G3m marker), IGHG3 gene (**Figure 7.3**), located on chromosome 14 at 14q32.33, which encodes IgG_3, and was recognised by sera from patients CC005 and CC014.

The IGHG3 gene is protein coding and has been shown to be associated with disease including heavy chain disease (HCD) and gamma heavy chain disease. Heavy chain disease is a lymphoproliferative disease whereby the variant monoclonal Ig heavy (H) chain fragments are found in serum or urine. It has been shown that the gene for the gamma-3 chain had undergone extensive NH_2-terminal deletion (Alexander *et al,* 1982). Gamma Heavy chain disease occurs due to an overproduction of the heavy chain of antibodies leading to their abnormal production. This disease mostly affects older adults and shows similarity to malignant lymphoma in symptoms where they are apparent.

Five super pathways were identified as having IGHG3 involved in them, these are the classical antibody-mediated complement activation, Fc gamma receptor (FCGR) dependent phagocytosis,

immune system, immune response, Fc epsilon RI pathway and IL4-mediated signaling events (**Figure 7.4**).

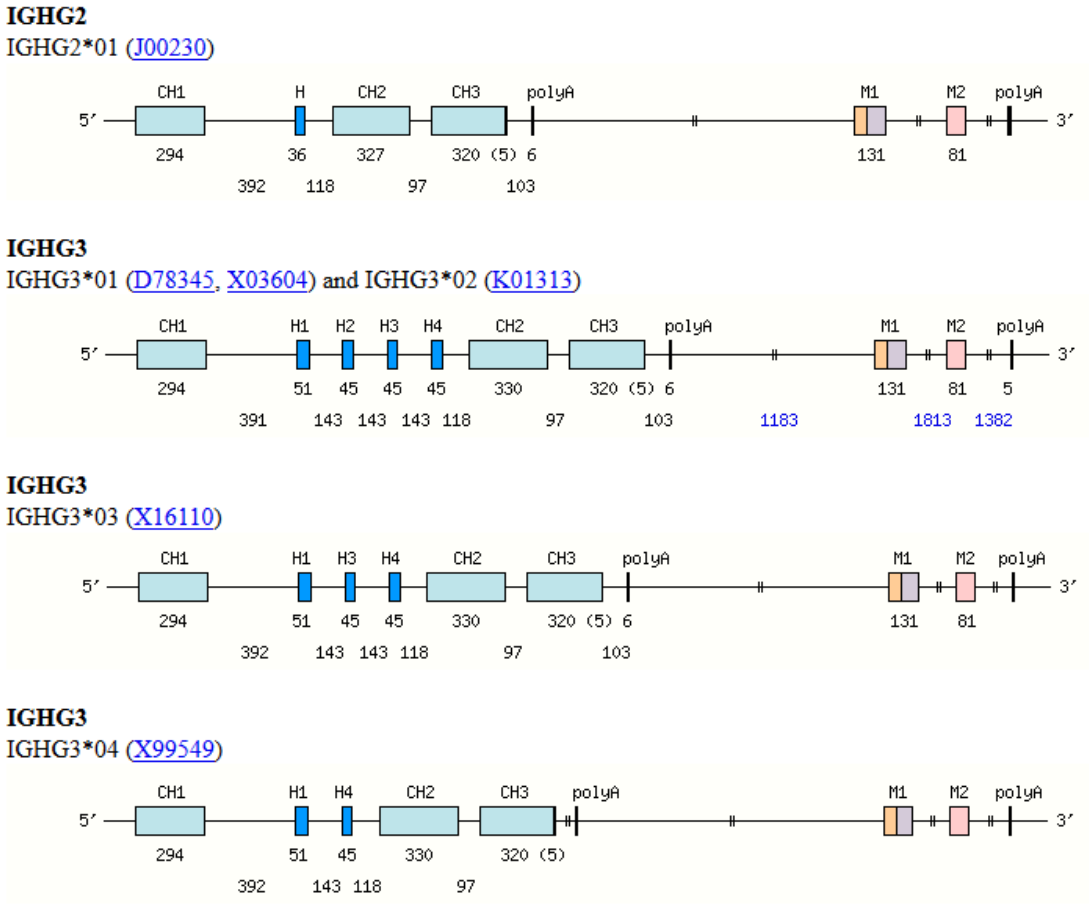


Figure 7.3 Human immunoglobulin constant genes IGHC represented on a single line. Image taken http://www.imgt.org/IMGTrepertoire/LocusGenes/exon-intron/Human/IGH/IGHC/Hu_IGHCorg.html (Lefranc & Lefranc, 2001). The line drawings depict the human immunoglobulin constant genes IGHG2 and IGHG3, cDNAs within which were identified by our SEREX studies. Large exons are not to scale. Upper line indicated the length of the exons, lower line the length of introns, are in base pairs. Numbers in parenthesis are the IMGT/LIGM-DB accession numbers.

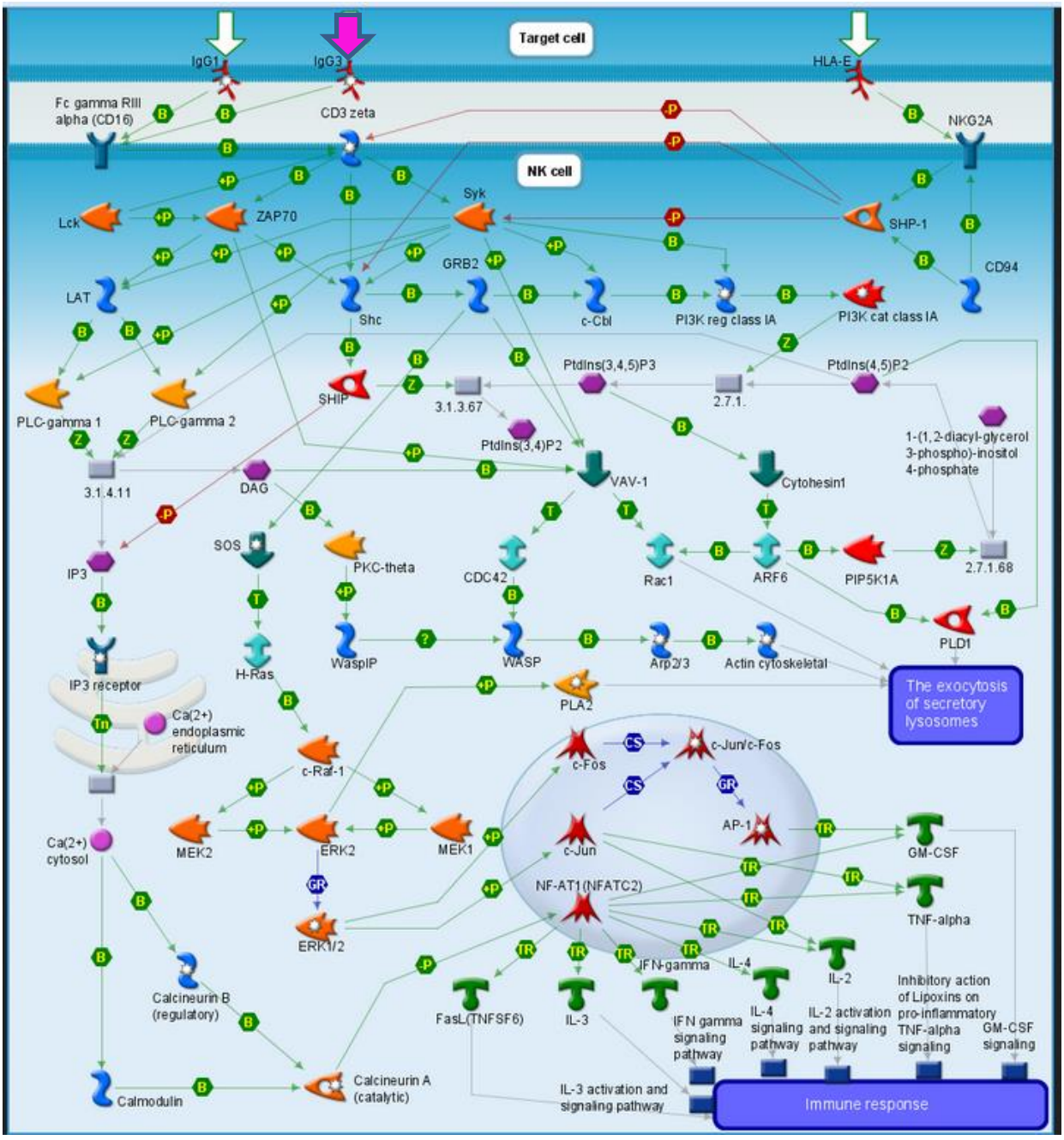


Figure 7.4. Immune response shown in terms of CD16 signaling in NK cells. Taken from http://pathwaymaps.com/maps/2249_map.png. Signalling via IgG3 in indicated by a pink arrow with a blue outline at the top of the image**.**

IGHG3 has three functional partners as identified by The Human Gene Compendium developed by the Weismann Institute (http://www.genecards.org/). The three most likely partners are **(A)** phosphoinositide-3-kinase, regulatory subunit 1 (alpha) (PIK3R1). Phosphoinositide 3-kinases are a family of lipid kinases capable of phosphorylating the 3'OH of the inositol ring of phosphoinositides. They are responsible for coordinating a diverse range of cell functions including proliferation, cell survival, degranulation, vesicular trafficking and cell migration. To date IGHG3 has been found most frequently to play a role in the regulation of insulin; **(B)** Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Alpha (PIK3CA) is the catalytic subunit, which uses ATP to phosphorylate Phosphatidylinositol, Phosphatidylinositol 4-phosphate and Phosphatidylinositol 4,5-bisphosphate to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 plays a key role in recruiting PH domain-containing proteins to the membrane and activating signal cascades involved in cell growth, survival, proliferation, motility and morphology as well as cell signalling in response to a range of growth factors (Bunney & Katan, 2010). PIK3CA has been found to be oncogenic and has been implicated in cervical cancers (Ma *et al*, 2000); and the final most likely partner for IGHG3 is **(C)** IGHG1 Immunoglobulin Heavy Constant Gamma 1 (G1m Marker).

In 2001 Lawrence *et al* described the increase in IGHG3, a novel immune function gene, in inflamed colonic tissue (both ulcerative colitis and Crohn's disease) as one of several genes whose expression had not previously been linked to inflammatory bowel disease. To do this they had examined global gene expression profiles using DNA microarrays. In total about 20% of the 170 differentially regulated genes were common to both forms of irritable bowel disease, probably reflecting common events secondary to inflammation. This would fit with the idea that immune reactive colon cancer has an inflammatory element. Indeed Murphy *et al*, 2000 paper had demonstrated an association between the occurrence of an inflammatory reaction, clearance of micrometastases and prognosis in patients

with immune reactive Dukes B colon cancer. IGHG3 has subsequently been shown to be overexpressed in non-squamous non-small cell lung cancer (Remmelink *et al*, 2005) breast cancer (Bin Amer *et al*, 2008), prostate cancer (Ledet *et al*, 2012) and malignant mesothelioma, the latter as part of a panel of nine autoantigens which may aid disease detection when using protein microarrays. The role of IGHG3 expression in cancer cells remains unclear although there have been some reports that IgG secreted by cancer cells can directly promote the growth and survival of cancer cells (Wang *et al*, 2013) including colon cancer cells (Barbera-Guillem *et al*, 2002; Qiu *et al*, 2003).

UOB-COL-2-cl and UOB-COL-2-c2, both recognised by CC014 sera, respectively, encoded part of the immunoglobulin heavy constant gamma 2 (IGHG2) gene located on chromosome 14 at 14q32.33. This is an important paralogue of IGHG3 although not, until now, implicated directly as having a role in any cancer and with little to add about its function except that it is a component of the IgG2 immunoglobulin providing the heavy chain.

The three other interesting and previously identified genes which we found through the immunoscreening of the testes cDNA library with immune reactive Duke's B colon cancer were CYB5R3, RPL37A and SLC34A2. CYB5R3 gene encodes the NADH-cytochrome b5 reductase 3 protein. This protein is membrane bound (i.e. ER and mitochhondria) in somatic cells and functions by desaturating and elongating fatty acids, in drug metabolism and in cholesterol biosynthesis. With regards to a possible role in cancer, CYB5R3 has been shown to be a putative biomarker of cancer migration in mice with a mutated K-ras in whom lung tissues were analysed (Lee *et al*, 2009). In breast cancer CYB5R3 has been shown to be downregulated, negating its' ability to detoxify reactive hydroxylamine metabolites of known mammary carcinogens (Rhoads *et al*, 2011)

Ribosomal protein L37a (RPL37A) is a ribosomal protein, and ribosomes are the organelles which catalyse protein synthesis. RPL37A belongs to the L37AE family of ribosomal proteins, it has a C4-type zinc finger-like domain and resides in the cytoplasm. RPL37A that has been found to be upregulated in high grade astrocytomas (MacDonald *et al*, 2007) and to have a general association with lifetime glioblastoma survival and overall glioblastoma survival (P-value < 0.05 in both cases)(Serão et al, 2011). In breast cancer RPL37A expression can be used in conjunction with MTSS1 and SMYD2 was shown to be able to predict the response of breast cancer patients to neoadjuvant doxorubicin and cyclophosphamide (Baros Filho *et al*, 2010).

Solute carrier family 34 member 2, the protein encoded by SLC34A2 gene is a membrane bound pH-sensitive sodium-dependent phosphate transporter (NaPi-2b) which belongs to the SLC34A transporter family. As a member of the sodium-dependent phosphate transporter family, NaPi2b is primarily involved in the maintenance of phosphate homeostasis in the human body. The role of NaPi2b in oncogenic transformation and malignant growth is not well understood. However, it has been reported to be overexpressed in 70-90% of epithelial ovarian cancers (Gryshkova *et al*, 2011). SLC34A2 has been found to be upregulated in a number of different well-differentiated tumours in comparison to poorly differentiated ones (Shyian *et al*, 2011), suggesting that it may play a role in cell differentiation processes during carcinogenesis. SLC34A2 is also shown to be involved in the development of breast cancer in an independent study (Chen *et al*, 2010). These suggest that the SLC34A2 gene could be an efficient diagnostic and therapeutic marker in cancer. In our study, SLC34A2 was recognised by both CC014 and CC005 patient sera which makes it an gene of interest for further investigation in relation to Duke's B (Chapter 8 future directions).

We immunoscreened a testes cDNA library to enhance our chances of finding a cancer-testes (CT) antigen. CT antigens are particularly desirable for immunotherapy because they are expressed in

cancer tissues and only in immune protected sites which lack MHC class I. The lack of MHC class I means they cannot present self-antigens to the immune system. Immunologically protected sites include mostly important reproductive tissue such as the placenta and testes and exposed but essential tissues such as the eyes. It's important that when a fleck of dust goes in the eye you don't have an immune response that renders you blind. Some antigens have expression limited to non-essential healthy tissue such as the breast. For women with cancer, if the option is life-saving cancer treatment and the risk of auto-immune response to the breast tissue then this may be considered a worthy option. Of course all patients would need to provide informed consent prior to treatment, especially in clinical trials and in all clinical situations patients have the right to refuse treatment except if they are children or vulnerable adults.

Most antigens identified by SEREX are overexpressed and not mutated. A growing interest in identifying mutated proteins in cancer as these would act as cancer unique targets. However there are a vast number of different mutations and so focus would remain on the most commonly mutated antigens such as p53 nucleotide 273 for example.

We also immunscreened our testes cDNA library with sera from three different Duke's B reactive patients to maximise not only the discovery of new antigens specific to Duke's B reactive disease but also to show very quickly whether any of these antigens were recognised by two or more patients.

We found a number of antigens which were recognised by more than one patient serum and we subsequently screened the same antigens with sera from healthy donors. Most of the antigens were recognised by at least some of the eight healthy volunteer sera with few exceptions (Table 4.3). However, the sera was used on two occasions only and meant that there was a significant background noise and therefore, the high chance of false positives. Further use of the sera should clear out the

background staining and lead to more conclusive results. Within our time-frame we were unable to screen the fifteen antigens with sera from patients with non-rective Duke's B. This should remain a future direction and would complete our investigations.

The testes cDNA used to make the library was commercially purchased however the frequency of phage which lacked an insert was quite high (around 30%). To determine whether the heavy chain IgG antigens identified through the immunoscreening of the library were due to B cell comtamination within the library Ms. Payalben Savaliyva, a fellow PhD student in Dr Guinn's group, immunoscreened the testes cDNA library with anti-IgG/secondary antibody. However no positives were found following the immunoscreening of 300+ pfus. This suggests that the heavy chain fragments (IGHG3 and IGHG2) were not contamination but reacted with by patient sera reflecting the immune/inflammatory component of Duke's B reactive disease, although this requires further investigation to validate it (discussed in Chapter 8 Future directions). We also checked the size of the cDNA inserts in the library we prepared and found a range of insert sizes indicating the library represented the breadth of testes cDNAs and was not limited in some way during its amplification.

RT-PCR analysis was used to see whether any known antigens were overexpressed by colon cancer samples and to optimise this study we initially analysed SW480, K562 and HL60. Only data from SW480 are shown in results chapter 6.

CHAPTER 8 Future directions

To complete this study, the most important next steps would be to complete tertiary immunoscreening with healthy donor and non-reative Dukes' B colon cancer sera and determine which of the antigens identified are solely recognised by sera from patients with Dukes' B reactive disease.

Despite our immunscreening of a testes cDNA library with sera from patients with Duke's B reactive disease we did not find any CT antigens, as far as we are aware. Further immunoscreening of the library to reach the agreed standard of immunoscreening 10^6 pfus may have helped us identify some CT antigens (Sahin *et al*, 1995). With the antigens we have identified we would want to investigate whether they are expressed by colon cancer tumour cells from patients with reactive and non-reactive disease. We would expect tumour antigens to have expression that is either restricted to tumour tissue or elevated in tumour tissue compared to healthy tissue. Most CT antigens are expressed at very low levels in healthy tissues if at all, so $1/100^{th}$ of the levels in cancer tissues. Some people believe any expression in healthy tissues, even at much lower levels, negates their title as CT antigens. However some of the most tissue restricted CT antigens show very small amounts of expression in healthy tissue, which is not immune protected such as PASD1 in pancreatic tissue (Guinn *et al*, 2005) and SSX2 in thyroid tissue (Tureci *et al*, 1996).

We would also have liked to analyse the expression of known tumour and CT antigens in patients with Duke's B colon cancer (reactive vs. non-reactive disease) compared with healthy colon tissue. We had started to optomise RT-PCR for a number of known CT antigens but many still needed further work to ensure we could reproduce results consistently with nice clean PCR products. WE

had performed our tests on SW480, K562 and HL60 but realised for some of the PCR reactions, new primers and slightly different PCR conditions were required. Once RT-PCR was optimised we had hoped to perform quantitative RT-PCR with the help of collegeaues at the Genomics Centre, King's College London. We would also suggest that progress in this project includes the immunocytochemistry confirmation of the antigens identified in patient tissue (FFPE sections). This wuld have followed tertiary screening when a short-list of Dukes' B reactive disease antgens were available. In addition enzyme linked immunosorbance assay (ELISA) could be used to detect the antigens or the antibodies to the antigens in patient sera. In light of the identification of IGHG3 and IGHG2 we could have analysed patient sera for the presence of cytokines to help us understand the cytokines playing a role in Dukes' B reactive disease. Mostly simply this could be done by ELISA.

We would be keen to determine whether RPL37A is a biomarker for survival in Dukes' B colon cancer and indeed all colon cancer patients. This could be investigated using cDNA microarrays which have been used to analyse Dukes' B tissue to determine whether RPL37A and survival in patients show an association.

Further analysis of the as yet unidentified sequences identified by secondary immunoscreening has recently led to the realisation that UOB-COL-5 encoded SLC34A2. Further analysis of the remaining, and as yet unidentified sequences, is likely to lead to the identification of these remaining sequences as our understanding of the human genome and it's availability for *in silico* analysis continues to expand.

There are a number of different options for immunotherapy. These range from the modification of tumour cells to enhance MHC expression, tumour antigen expression or more typically cytokine expression for adoptive transfer of whole cell vaccines. Adoptive therapy of immune system cells

such as T cells or NK cells where these cells are isolated, purified and expanded ex vivo to has been shown to be effective where numbers of tumour specific adoptively transferred cells are high enough (reviewed in Hinrichs and Rosenberg, 2014). Modification of TCR receptors to recognise specific epitopes presented in the context of MHC on cancer cells have been used by a number of groups to enhance the effectiveness of adoptively transferred T cells (Stauss *et al*, 2008). Any of the antigens we have identified could be used as targets for immunotherapy, hopefully stimulating immune responses in patients with Duke's B non-reactive disease or enhancing responses in patients with reactive disease who may be part of the small percentage that do not clear their micrometastses after surgery. Antigens identified by SEREX would need to be validated. This would require the demonstration of whether they were expressed in healthy tissues which would impact cancer specific targeting. We would also need to determine the frequency of expression in patients to determine whether targeting this antigen would be valid and at what stage of malignancy expression is found. As two of the antigens were IgG heavy chains it would be interesting to determine whether these proteins are being secreted into the serum or blood of patients and could allow early detection or act as biomarkers for disease – differentiating patients with reactive disease from those with non-reactive disease without the need to take bone marrow aspirates for micromestastases.

The depletion of regulatory T cells ($CD4^+$ $CD25^+$) using anti-$CD25^+$ antibodies has been shown to enhance anti-tumour responses (Whelan *et al*, submitted) and it is likely immunotherapy will not only need to be used in conjunction with conventional therapies but also in synergy with other immunotherapy protocols to achieve full tumour clearance.

CHAPTER 9 References

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Appendix I Publications

Cancer Gene Therapy (2013) 20, 157–168

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REVIEW

New targets for the immunotherapy of colon cancer—does reactive disease hold the answer?

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Colorectal cancer (CRC) is one of the most commonly diagnosed cancers in both men and women, posing a serious demographic and economic burden worldwide. In the United Kingdom, CRC affects 1 in every 20 people and it is often detected once well established and after it has spread beyond the bowel (Stage IIA–C and Stage IIIA–C). A diagnosis at such advanced stages is associated with poor treatment response and survival. However, studies have identified two sub-groups of post-treatment CRC patients - those with good outcome (reactive disease) and those with poor outcome (non-reactive disease). We aim to review the state-of-the-art for CRC with respect to the expression of cancer-testis antigens (CTAs) and their identification, evaluation and correlation with disease progression, treatment response and survival. We will also discuss the relationship between CTA expression and regulatory T-cell (Treg) activity to tumorigenesis and tumor immune evasion in CRC and how this could account for the clinical presentation of CRC. Understanding the molecular basis of reactive CRC may help us identify more potent novel immunotherapeutic targets to aid the effective treatment of this disease. In this review, based on our presentation at the 2012 International Society for the Cell and Gene Therapy of Cancer annual meeting, we will summarize some of the most current advances in CTA and CRC research and their influence on the development of novel immunotherapeutic approaches for this common and at times difficult to treat disease.

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Keywords: colon cancer; SEREX; cancer-testis antigens; tumor antigens; reactive disease; immunotherapy targets

THE GLOBAL CHALLENGE

'You have cancer'—the diagnosis that millions of people around the world hear every year and also the second leading cause of death worldwide. The number of new cancer cases each year is gradually increasing and the mortality rate is expected to rise from 7.6 million in 2008 to over 17 million in 2030.[1,2] On average, one of every three people is expected to experience some type of cancer in the course of their lifetime and this frequency is expected to increase[3] as the population ages. The World Health Organization reports that an increasing percentage of all newly diagnosed cancer cases annually occurs in low- and middle-income countries and this is attributed to a wide range of behavioral, genetic and environmental risk factors.[4] Nevertheless, the increase in the average life expectancy and the adoption of an unhealthy lifestyle (such as smoking, physical inactivity and poor diet) worldwide have also contributed to the rise of a new trend in the cancer demography. Newly diagnosed cancers from the low- and middle-income countries today account for 451% of the total number and their share in the global burden is expected to continue to increase with the growth and aging of the population.[4] In addition, the lack of resources for early cancer detection and effective treatments in the developing world contributes to an increase in cancer-related deaths.

Today, some of the most commonly diagnosed cancers both in economically developing and developed countries include prostate, breast, lung and colorectal cancer (CRC)—'the big four'—accounting for nearly 50% of the total cases diagnosed.[5] Cancer is a global challenge, opposing a serious demographic and economic burden with worldwide economic costs estimated to be as high as £572 billion per year. These alarming statistics resulted in the development of national strategies and action plans for cancer control including prevention, early detection and effective treatment.[6] However, the demand for the development of new approaches to cancer screening and therapy is recognized globally and brings the research attention on these aims sharply into focus.

COLORECTAL CANCER

CRC is one of the most commonly diagnosed cancers worldwide. It affects the bowel and the rectum and is rare in people under 40, with almost 85% of cases being diagnosed in persons over 65 years of age.[6] Statistics show that men and women are affected equally, while it is the third most common type of cancer in men (after prostate and lung cancer) and the second most common cancer in women (after breast cancer).[6] One in every 20 people in the UK develops CRC with only half of them surviving beyond 5 years, mainly because it is often detected once well established and after it has spread beyond the bowel. The disease stage at the time of diagnosis governs both the choice of treatment and the prognosis. CRC is staged to reflect how far the cancer has spread and whether or not it has reached nearby structures such as lymph nodes or distant organs. The most commonly used staging system for CRC is that of the American Joint Committee on Cancer,

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also known as tumor nodes metastases system.[7] It describes three key pieces of information: 'T'—how far has the primary tumor grown; 'N'—the extent of spread to nearby lymph nodes; and 'M'—describes whether the cancer has metastasized. The information from the T, N and M is combined to determine the cancer stage grouping from Stage I (the least advanced) to Stage IV (the most advanced). Two of the older staging systems include Duke's[8] and Astler–Coller[9] but these are very rarely used today.

Stage I (A–C) (Duke's A–C) CRC is reported to be an asymptomatic malignancy, developing slowly by the progressive accumulation of genetic mutations within precancerous bowel lesions and polyps. Diagnosis at this stage reduces the risk of death from CRC, giving 90% chance of survival beyond 5 years[10,11] and significantly low levels of disease recurrence. However, most cases of CRC are detected once the cancerous cells have moved beyond the middle layers of the colon (Figure 1). This is classified as Stage IIB (Duke's B) and is one of the most commonly diagnosed forms of CRC. Currently, the course of treatment for CRC patients is fairly similar regardless of the significant differences in the biological features of each CRC case. Usually, the most effective approach is tumor resection, followed by chemo- or radiotherapy for Stage III and sometimes for Stage II CRC patients. However, recent studies suggest that not all Stage III patients benefit from these therapies and that 25% of Stage II cases are under treated.[12,13] Furthermore, a number of non-aggressive tumors are frequently overtreated, leading to the patient experience of unnecessary and severe side effects. Methods such as, fecal occult blood test, sigmoidoscopy, colonoscopy, virtual colonoscopy and double contrast barium enema offer improvements in the detection rates of CRCs.[14] However, their diagnostic value is limited with regards to costs, risks, lack of sensitivity especially in early stages and inconvenience to the patient.[15] Therefore, the focus remains on developing efficient methods for the early detection of CRC such as the identification of early disease biomarkers which could be used in non-invasive (urine and blood serum) tests. Such molecular biosensors for CRC would enable widespread screening alongside general health examinations and may further reduce the mortality rate associated with late stage detection of CRC.[16]

Murphy et al[17] suggested that there are two sub-groups of patients with Stage IIB CRC—those with good treatment outcome (reactive disease) and those with poor treatment outcomes (non-reactive disease). Whether the difference between these two groups of patients can be determined by differences in humoral responses warrants further investigation and provides the basis of our own current studies. Tumor biomarkers offer an opportunity to translate unique CRC biological features into diagnostically pertinent information and would enable persona-lized treatments, which could inform conventional and immu-notherapeutic interventions. This would enable discerning treatment strategies for aggressive and non-aggressive cancers and the clear 'up front' distinction of reactive from non-reactive disease.

In part, to address this need, researchers are investigating immune responses in cancer patients to identify new immu-notherapy targets and biomarkers. They are hoping to identify and evaluate tumor-associated antigens (TAAs) and cancer-testis antigen (CTAs) that could prove to be efficient diagnostic, prognostic or immunotherapeutic targets in CRC.[18] Recent developments in the fields of genomics and proteomics have greatly contributed to these studies, enabling the identification of multiple potential antigens within a single experiment.[19] Techniques, such as DNA microarray analysis, protein microarrays, peptide-major histocompatibility complex (pMHC) tetramers, serological identification of antigens by recombinant expression cloning (SEREX), serological proteome analysis (SERPA) and peptide elution from MHC for mass spectrometry analysis are now commonly used to evaluate the expression profiles of genes and proteins as well as antigen recognition within different types, sub-types and stages of cancer. Their application in a number of studies conducted on acute myeloid leukemia (AML),[20,21] diffuse large B-cell lymphoma,[22] lung cancer[23] and osteosarcoma[24] have already proven successful and have led to the discovery of a large panel of antigens with prognostic and immunotherapeutic relevance. It has also provided an insight into the biological complexity and individuality of each cancer case, demonstrating considerable heterogenicity among patients and within tumor types. Therefore, genomics, proteomics or a combination of multiple methods could aid the discovery of novel biomarkers specific for

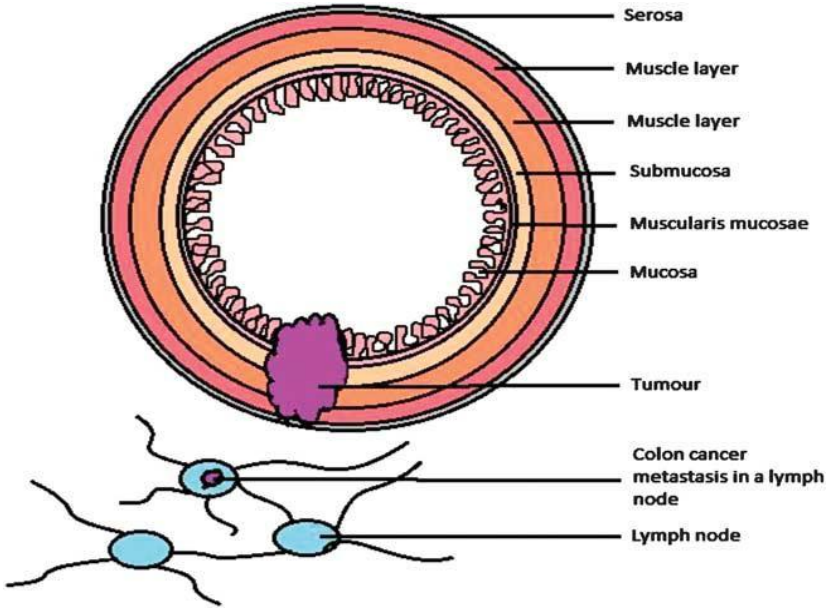

Figure 1. Shows a malignant primary tumor that has moved beyond the middle layers of the colon and has also metastasized to a nearby lymph node. These are stages IIB (Duke's B) onwards.

CRC and contribute to the development of personalized therapies, which would maximize efficiency and minimize side effects for the patient.

THE SEARCH FOR TUMOR ANTIGENS SPECIFIC FOR CRC

The desire to identify TAAs has inspired and attracted researchers for more than four decades. Most efforts were driven by the aim to uncover specific epitopes on cancer cells, which could elicit immune responses in the autologous host.[25,26] In the 1970s, a method known as 'autologous typing' was used successfully to identify a number of antigens including alpha fetoprotein (in hepatoma and germ cell tumors), carcinoembryonic antigen (CEA)
(in gastrointestinal cancers), prostate-specific antigen (in prostate cancer), CA125 (in ovarian cancer) and AU (in melanomas).[27,28] This novel serological technique offered a substantial improvement over existing methods as it enabled the analysis of the cellular (T-cell defined) anti-tumor human immune responses in an autologous manner where only tumor-specific antigens could be recognized.[27] However, autologous typing did not completely fulfill the original hopes for its success because of several limitations. The major disadvantage arose from its reliance on cultured tumor cell lines and not all tumor types could be propagated ex vivo to allow autologous typing to be performed. Furthermore, only a small fraction of the total number of patients was found to have demonstrable levels of autologous antibody with specificity for cell surface antigens on their tumor.[25,26] In the cases when the technique was successful and a tumor antigen was identified, the low titer of antibodies that were often detected made any further biochemical or molecular characterization of the antigens almost impossible. However, the method of autologous typing contributed to the identification of a number of human tumor antigens, which were categorized into four classes: differentiation antigens—for example, gp-100; mutational antigens—for example, abnormal forms of p53; retroviral antigens—Epstein–Barr virus and human papilloma virus; and CT antigens.[26]

CT antigens

CTAs were classified as TAAs with restricted expression in testis, placenta and various types of cancer.[26] In the field of cancer serology they were quickly identified as the ideal targets for tumor-specific immunotherapeutic approaches.[29–33]

The selective expression pattern of CTAs is considered to be a consequence of gene activation by DNA demethylation[34,35] and histone post-translational modifications[36] both occurring constitutively in the testis, but also in tumor cells.[35] Some CTAs have been found to be expressed in healthy tissues such as liver, pancreas and spleen but at a significantly lower level (o1%) when compared with their expression in testis.[31] Around 70 families of CTAs have been identified to date and further classified as X-CTAs and non-X-CTAs depending on the chromosomal location to which the genes are mapped.[37] The genes for distinct X-CTAs have been previously reported to encode for different antigenic peptides that are presented with HLA class I or HLA class II allo-specificities, eliciting both cell-mediated and humoral immune responses.[38] The blood–testis barrier and the lack of HLA class I expression on the surface of germ cells prevents the cells of the immune system from interacting with the CTAs expressed there.[39] It has been suggested that as the B and T cells have not been previously challenged by CTAs the immune response should be able to recognize CTAs as non-self structures when expressed on cancer cells[39] circumventing the need to break tolerance. These findings suggest that CTAs can be viewed as promising molecular targets for the development of immunotherapeutic interventions for specific cancer types without the possibility of triggering autoimmune responses. The identification of CTAs specific for a particular type of cancer is a promising approach for the development of peptide or recombinant full-length protein anti-cancer vaccines and for antigen-specific adoptive T-cell transfer as part of cancer therapy.[40] In that respect, NY-ESO-1 (a CTA with strong immunogenicity still detectable at sera dilutions of 1 in 40 000[41]) has been of interest in relation to the development of cancer vaccine trials. Several ongoing trials have been based on this antigen alone or its use in combination with an immune enhancer are currently ongoing.[42,43] However, the expression of other CTAs within various types of cancer and at different disease stages appears to be heterogeneous and further investigation is required for the identification of effective immunotherapeutic targets.

Although, CTA expression is generally correlated with tumor progression and immunogenicity in various types of cancer, little is known about this in relation to CRC. In addition, CTAs were often found to be poorly expressed (low level, heterogeneous expression) in CRC[44] highlighting the need for the identification of further immunogenic CTAs involved in CRC development. Such proteins would be candidates for cancer-specific therapy trials and may provide effective biomarkers for diagnosis, prognosis and monitoring of CRC disease progression.

Serological identification of antigens by recombinant expression cloning

The limited success of autologous typing demonstrated the need for a more comprehensive experimental approach for the identification of TAAs. It was not until the mid-1990s when a new autologous immunoscreening technique was shown to circumvent some of the limitations of autologous typing. This method was called SEREX.[45] It allowed the identification of numerous TAAs based on their recognition by antibodies in diluted pre-cleared autologous patient sera and in a small number of experiments. The advantages of SEREX were quickly recognized and a substantial pool of data encompassing serologically relevant antigens within cancer began to collect. SEREX was capable of identifying immunoglobulin G antibody responses to both highly (NY-ESO-1) and weakly immunogenic TAAs.[46,47] Approximately one-third of these antigens were novel and referred to as SEREX-defined antigens. In addition, sera was shown to be able to detect CD8 ^{þ} T-cell recognized TAAs, including NY-ESO-1,[41] MAGE-1 and tyrosinase[41,45,48] as well as TAAs recognized by immunoglobulin G antibodies, which are also known to require CD4 ^{þ} T-cell help (NY-ESO-1).[45,46,48–51] These findings demonstrate that the cellular and humoral immune system work in concert and are both stimulated by TAAs in the case of cancer. Furthermore, several antigens (NY-ESO-1, MAGE-1 and hMena)[52] that elicit humoral as well as cell-mediated immune responses have also been successfully identified through SEREX. Studies have investigated CD8 þ and CD4 þ T-cell recognition of SEREX-defined antigens and the data showed that co-immunization with such proteins in combination with a cytotoxic T lymphocyte (CTL) epitope enhanced CD8 ^{þ} induction in a CD4 ^{þ} T-cell-dependent manner.[53,54]

The continuously increasing number of SEREX antigens led to the creation of a Cancer Immunome Database, a free repository for the antigens identified by this serological approach.[55] The success of SEREX lies in its reliance on the construction of a cDNA library from human tissues (cell lines, primary tumor or normal donor testes) and their expression in a prokaryotic system.[45] Immunoscreening with SEREX permits quick and effective extraction, processing, sequencing and subsequent molecular analysis of the protein of interest as both the TAA and its coding cDNA are present in the same plaque. However, the use of SEREX has several limitations. It has been previously reported that SEREX-defined antigens are predominantly nuclear proteins which are often transcription factors[41] and/or ubiquitously expressed.[55,56] As such, they rarely show evidence of mutations or other structural

abnormalities and are often weakly immunogenic, incapable of eliciting and sustaining strong humoral immune responses.[25] In addition, phage can only express proteins in their primary structure, which may cause the failure to detect antigenic sequences consequent to eukaryotic post-translational modifications.[57] Regardless of the limitations, SEREX-defined antigens are already being investigated as diagnostic markers (testis-specific protease (TSP50) in ovarian and CRCs)[58] and as immunotherapeutic targets (OVA66 in ovarian cancer,[59] KIF20A in pancreatic cancer[60]).

Serological proteome analysis

SERPA is a powerful tool used for the identification and validation of immunogenic TAAs. Similar to SEREX, it uses the antibody repertoire contained within the sera of a cancer patient to successfully identify TAAs.[61] In comparison to SEREX, SERPA does not require the use of a cDNA library, making this method less time consuming and less labor intensive. Furthermore, SERPA is better suited for the detection of possible post-translational modification and protein isoforms as it relies on the separation of complex mixtures of proteins extracted from cell cultures or tumors. The separation is performed via the use of a two dimensional gel electrophoresis (2-DE).[62] A number of TAAs have been identified to date using SERPA in various types of cancer including lymphoma,[63] renal cell carcinoma,[64] ovarian cancer,[65] and CRC.[66] These studies have established the specificity of SERPA and its great potential in uncovering immunogenic TAAs and identifying tumor markers. However, similar to SEREX, most of the antigens identified by SERPA are predominantly weakly immunogenic intracellular proteins and very rarely membrane-associated ones.[67,68] This and the finding that a number of antigens found by T-cell cloning have also been found by SEREX suggests that most antigens induce B and T-cell responses,[69] although not necessarily to the same epitope(s). In addition, SERPA has demonstrated a drawback associated with the use of 2-DE: low abundance (such as regulatory and signal transduction proteins and receptors[70]), hydrophobic or insoluble proteins (such as membrane proteins[70]) are inherently difficult to detect.[67]

SEREX and SERPA are two methodologies that seem complementary to each other as they identify two different sets of antigens (SEREX for the identification of antigens with altered expression; SERPA for the identification of antigenic proteins resulting from post-translational modifications). Therefore, the identification and validation of CRC-related CTAs using SERPA and SEREX independently or as a combined approach is worthy of further consideration.

Recombinant antigen expression on yeast surface (RAYS)

RAYS is another serological strategy applied to the process of discovering immunogenic CTAs. RAYS permits the expression of immunogenic proteins on the surface of yeast allowing for a more natural folding of the protein and partial glycosylation (by virtue of it being a eukaryotic system).[57] This permits the analysis of proteins in their natural conformation when compared to the prokaryotic expression utilized by SEREX. This method has demonstrated specificity and sensitivity for the detection of an antibody response to a conformation-dependent epitope— the CRC antigen A33. Therefore, the CTAs that have escaped detection because of the fact that they elicit immune responses only after undergoing the appropriate post-translational modifica-tions could potentially be identified via RAYS. To date, RAYS has allowed the confirmation of antigen immunogenicity through screening of eukaryotic cDNA expression libraries derived from pancreatic cancer[71] and prostate cancer. [72] RAYS offers a less time-consuming analysis of the serological autoreactivity in cancer patients and it has provided an effective anti-cancer vaccine platform (recognizing NY-ESO-1) in prostate cancer patients.[72]

However, its further development is required to allow the detection of novel target antigens (such as is achieved with SEREX or SERPA) and its application in CRC requires further analysis.

Serum antibody detection array

To evaluate the seroreactivity of a number (or a panel) of SEREX-defined antigens in a particular type of cancer, a spot immunoassay, known as serum antibody detection array, has been successfully developed and utilized.[73] Although, several antigens have been evaluated in cases of colon cancer (MAGE-A3, SSX2 and NY-ESO-1[73]), additional improvements in the sensitivity of serum antibody detection array are still required.

Protein microarrays

Protein microarrays allow the rapid and easy detection of tumor antigens using patient sera.[74,75] Recent studies have demonstrated the efficiency of the technique in the rapid identification of immunogenic membrane-based TAAs with high reproducibility of the experimental analyses of lung and brain[74] and ovarian[76] cancers. In this context, protein microarrays has a great advantage as this methodology allows the construction and simultaneous analysis of a large panel of candidate tumor biomarkers (~9,000). However, it should be noted that although protein microarrays have vast screening potential they are limited to defined proteins from an albeit not insubstantive pool.

Understanding the molecular interactions between T-cell receptors on CTL and peptide/MHC class I complexes on tumor cells is an essential tool for the development of immunogenic vaccines.[77] Several such vaccines have already been designed based on TAA epitopes and have been implemented in Phase I and II clinical trials for different types of cancer (human papilloma virus,[78] WT1,[79] human telomerase reverse transcriptase[80] and HLA-A24 þ HRPC[81] peptide vaccinations). The strength of vaccine-mediated immunological responses generally need to be enhanced and this will be much more feasible in patients in subsequent (Phase III) clinical trials who are likely to have less advanced disease. However, the results obtained from clinical trials to date warrants further investigation.

pMHC (tetramer) microarrays

The development of pMHC microarrays has allowed the rapid identification of antigen-specific populations of T cells in the peripheral blood of patients.[82] This approach has proven useful for epitope prioritization, and for the detection of multiple T-cell populations in cancer patients undergoing conventional treatments or tumor-associated peptide vaccine trials.[82,83] We are using the pMHC array to identify which epitopes are recognized by peripheral T cells from colon cancer patients during conventional treatment (Bonney et al., in preparation).

TUMOR PROFILING IN PATIENTS WITH CRC

A number of studies have been conducted over the last decade aiming to identify novel biomarkers that would prove to be efficient in CRC profiling as prognostic, predictive or therapeutic biomarkers. The recent improvements in proteomics and genomics methods has greatly aided this aim and has led to the identification of a number of CTAs and TAAs relevant to CRC. However, the clinical significance of only a small fraction of these potential markers in CRC has been evaluated to date.

CTAs in CRC

NY-ESO-1 has been recently studied in relation to CRC. It was demonstrated that some CTAs are capable of eliciting strong humoral and cell-mediated immune responses in some patients

with CRC.[84] However, its expression in CRC is often highly heterogeneous, when present, which poses an obstacle in the development of a generalized immunotherapy. As discussed earlier in this review, NY-ESO-1 has also been targeted in several vaccine clinical trials worldwide involving CRC patients. Recently, a new Phase I trial of a fusion protein vaccine is being organized targeting solid tumors expressing NY-ESO-1 and this investigation includes Stage I–IV CRC.[85] In addition, NY-ESO-1 expression was shown to correlate with CRC stages and local lymph node metastasis[86] making it a potential prognostic biomarker for CRC.

TSP50 was originally identified as abnormally expressed in breast cancer cells[87] and has recently been studied for the first time in CRC patient samples. The expression of TSP50 was found to correlate with the clinicopathological characteristics and disease-specific survival for CRC patients.[88] Furthermore, the study demonstrated that TSP50 expression is highly specific for CRC as compared with colorectal adenomas and normal tissues, allowing the easy differentiation between them. TSP50 is an attractive predictive biomarker for poor survival in patients with early stages CRC (Stage I and II), but not in patients with advanced stage disease. As such, it is the only effective predictive biomarker reported to date for patients with early stage CRCs.[88]

CABYR is a calcium-binding tyrosine phosphorylation-regulated fibrous sheath protein and its expression was first identified in human spermatozoa ([89]; reviewed in Chiriva-Internati et al.).[90] Subsequent detection of CABYR in lung carcinoma and its absence in healthy tissues, led it to be considered as a novel CTA, which has been shown to have some immunogenic properties that could serve as a basis for the development of immunotherapy for cancer patients.[91] Additional studies on CABYR expression in brain,[92] hepatocellular[93] and other carcinomas[94] have shown that there are at least five different isoforms of this protein, which could have unique roles in the process of carcinogenesis. Following these discoveries a recent study has reported a frequent overexpression of CABYR a/b and c isoforms in CRC tumors when compared with adjacent normal tissues.[95] However, a more comprehensive investigation is required to determine whether CABYR expression correlates with tumor stages and is a suitable therapeutic vaccine candidate in CRC.

SPAG9 is another antigen found to be exclusively expressed in testis[96] that is a particularly attractive target for immunotherapy in epithelial ovarian cancer,[97] thyroid cancer[98] and in CML. [99] A recent study has investigated the expression of SPAG9 in CRC patients aiming to explore its possible role in colon cancer tumorigenesis and its effectiveness in eliciting a humoral immune response.[100] Interestingly, the study has reported a close relationship between SPAG9 expression and early stages of CRC development suggesting that it could serve as an early diagnostic biomarker for CRC patients. The investigation had also demonstrated that SPAG9 could have a key role in the tumor development and could also serve as a target for the development of immunotherapeutic methods.

Other CTAs, found to exhibit a strong correlation with CRC presentation are listed in Table 1.

TAAs in CRC

CD133 is a cell surface protein marker found on undifferentiated cancer cells that exhibit stem-like properties. These cells account for the propagation, growth and recurrence of AML[110,111] and CRC[112,113] and for the resistance of these cancers to current therapies. The functional importance of CD133 expression has been investigated in several studies in relation to the initiation and behavior of CRC.[113–115] The CD133 ^{þ} CRC stem cells are reported to have exhibited the ability to transfer cancer to a secondary recipient maintaining the same immunophenotype and the global gene expression profile of the primary tumor when compared with CD133 $^{-}$ cells.[113,114] Furthermore, several studies have clearly identified the correlation between CD133 expression alone[114,115] or in combination with other protein markers[116] with CRC patient survival and have revealed it to be a reliable prognostic marker. In combination with CD44 and CD166 (cell surface protein markers), CD133 expression has also been linked to the presentation of low-, intermediate- or high-risk CRC cases with the ability to distinguish between them at an early stage of the disease (stage II).[114,116] CD133 is a promising predictive and prognostic marker in the diagnosis of CRC and particularly of interest as it is applicable to the early stages of the disease. However, the presence of such cell surface markers on the CRC stem cells has not been investigated in relation to the underlying cause of the non-reactive type of CRC.

CEA is a TAA whose expression levels are often monitored pre- and post-treatment in CRC patients as they have been found to be indicative of cancer recurrence[117] and poor disease prognosis.[118,119] Patients with elevated post-treatment CEA expression levels are often monitored more carefully for relapse of CRC and for local or distant recurrence.[117,119] To date, several studies have investigated the potential of CEA as an immunotherapeutic target in cases of CRC. Different research strategies have incorporated CEA peptides or CEA mRNAs in dendritic cell vaccines[120,121] and in plasmid DNA vaccines[122] demonstrating that these vaccines are well-tolerated and have immune-stimulatory capacity in patients with CRC. However, the overall outcome of these studies indicated that additional vaccine modulation is necessary to attain significant clinical impact. More recently, a study to investigate whether the vaccination of toll-like

Table 1. Antigens determined to have potential as diagnostic, prognostic or immunotherapeutic targets in CRC

CTA/panel of CTAs	Chromosome location	Method of identification	First identified in	Potential uses in CRC
BCP-20 (FBXO39)	17p13.1	SEREX	CRC[101]	Candidate diagnostic and immunotherapeutic target[101]
PAGE4	Xp11.23	Database mining	CRC[102]	Predictive panel for liver metastasis[102]
SCP-1	1p13-p12	SEREX		
SPANX	Xq27.1	Differential display	Melanoma[103]	
MAGE-A4	Xq28	T-cell epitope cloning		Colon cancer vaccine therapy with peptide of MAGE-A4[104]
STK31	7p15.3	Three-step microarray analysis	CRC[105]	Candidate target for immunotherapy[105]
SSX	Xp11.2	Reverse transcription-PCR	CRC[106]	Co-expression as predictive marker for metastasis
MAGE	Xq28			Candidate targets for immunotherapy[106]
SSX2	Xp11.22	SEREX	Melanoma[107]	Candidate target for immune therapy[108,109]

Abbreviations: CRC, colorectal cancer; CTA, cancer-testis antigen; SEREX, serological identification of antigens by recombinant expression cloning; SSX, synovial sarcoma X antigen.

receptor activated dendritic cells can induce more potent CTL responses and antitumour activity in CEA transgenic mouse tumor models was published.[123] It has demonstrated that the combined activation of TLRs can lead to better maturation status of dendritic cells and can also induce more effective antitumour immune responses against CRC. However, additional investigation is necessary to evaluate the effectiveness of this approach in human models.

CLINICAL SIGNIFICANCE OF CTAS IN CRC

The CTAs that have already been identified within different types of cancer could serve as biomarkers for discerning aggressive and non-aggressive cancers and for predicting treatment outcome and relapse. Their expression patterns and clinical significance is still under investigation, but there are very promising early results. Some of their clinical applications are described in the followings sections, and could expand the list of potential CTAs in CRC.

Potential biomarkers for discerning reactive from non-reactive disease

The expression profile of CTAs in relation to treatment outcome in particular types of solid cancers has been previously studied on several occasions[24,52,58–60] but further investigation is necessary to compare these findings with CRC cases. In this context, a recent study has demonstrated that g-irradiation de novo upregulates the expression of various CTAs and MHC-I in a randomized fashion. Therefore, irradiation could be accounted responsible for the increased immunological response to certain tumors owing to the inflammation and cell damage it causes. This would be anticipated to cause the immune system to attend the site of damage, mop up cellular debris and present proteins including CTAs to the immune system.[124] These findings fit with demonstrations in leukemia that elevated tumor antigen expression at disease presentation is associated with improved survival.[125,126] Identifying and evaluating such TAA and CTAs would be beneficial for profiling individual tumors and for combining radiotherapy (or other cancer therapy approach) with immunization to maximize the effect of treatment in CRC. However, in order to design a combined treatment a thorough understanding about the mechanisms of initiating CTAs expression and the likely order of their expression is necessary.

The exact role of the numerous CTAs in relation to tumor response to various treatments still remains poorly defined. Particularly, in cases where adjuvant therapy is in order, it would be beneficial to have a panel of biomarkers to predict the likely success of the therapy. Several CTAs have been evaluated in gastrointestinal stromal tumor—with regards to recurrence, while levels of MAGE-A1, MAGE-A3, MAGE-A4, MAGE-C1 and NY-ESO-1 expression were investigated in response to imatinib adjuvant therapy.[127] This study demonstrated that CTA ^{þ} gastrointestinal stromal tumors had a significantly shorter recurrence free survival compared with negative cases. Furthermore, the expression of NY-ESO-1 and MAGE-A3 was associated with elevated resistance to imatinib and therefore, with continuous tumor progression. Luetkens et al.[128] have also showed that PRAME expression remains stable under imatinib treatment and correlates with decreased overall survival in patients with CML.

Similar findings have been reported in several studies of prostate and lung cancer, multiple myeloma, AML, liposarcoma and others.[129–134] These suggest an important role of CTAs in the pathophysiological behavior of different tumors in response to treatments. Further investigation of a panel of antigens associated with particular types of cancer and their relationship to either reactive or non-reactive disease is yet to be attempted. In this relation, TSP50 is the only known CTA to have been characterized as a biomarker for disease prognosis in CRC,[88] but its association with treatment outcome is still to be analyzed. Furthermore, investigation of the interrelationships between groups of CTAs in CRC and their expression profiles could lead to significant discoveries about the underlying cause of a particular treatment response. This could benefit the design of a multivalent cancer vaccine targeting several antigens rather than just a single one.

Potential biomarkers for survival prognosis

Several studies have investigated the role of the CTAs as biomarkers of prognostic value regarding patient survival. Elevated levels of expression of particular CTAs have been shown to correlate with poor survival prognosis, particularly in solid tumors. In contrast to expectations some antigens have been shown to have above average levels and have better survival rates in patients with hematological malignancies. For example, expression of SSX2IP in the presentation of AML has been shown to predict good survival in patients with no detectable cytogenetic rearrangements.[125] The elevated levels of antigens provide targets for improved immune responses in patients post-conventional (chemotherapy, radiotherapy) treatment, when there is cancer cell damage and inflammation (danger) signals stimulating an immune response to clear up dead and dying cells. Furthermore, the CTAs expressed on non-solid tumors (such as AML) are more accessible and 'easier to see' by the immune system as they are not hidden within heterogeneous layers of cancer cells (as seen in solid tumors).

The prognostic value of CTAs has also been evaluated in the presentation of osteosarcoma by gene microarray where the high expression of MAGE-A could predict distant metastasis and poor survival. For patients with and without MAGE-A expressing tumors, the 5-year survival rates were found to be 39.6% and 80% respectively.[24] Similarly, increased levels of WT1 (another CTA) expression have been shown to correlate to poor prognosis and relapse in pediatric AML after induction therapy.[135] A number of products of translocations have been used in routine labs, which detect minimal residual disease and can indicate impending relapse with high accuracy.[136] The CTA expression in relation to patient survival in CRC cases has been the focus of few studies and further investigation is required to establish the clinical significance of this relationship.[88]

Potential biomarkers for discerning aggressive from non-aggressive disease

A recent study on prostate cancer has demonstrated that several CTAs are preferentially expressed in either aggressive or non-aggressive disease.[137] Such biomarkers could be particularly useful in preventing the overdetection and overtreatment of potentially indolent CRC or the undertreatment of a more aggressive type of disease.

Potential immunotherapeutic targets

In 2005, cancer patients expressing NY-ESO-1 and LAGE-1 antigens entered a Phase I clinical trial on a plasmid DNA (pPJV7611) cancer vaccine,[138] demonstrating the effectiveness of CTAs as immunotherapeutic targets. Recently, the first clinical trial of cancer vaccine therapy with artificially synthesized helper/killer-hybrid epitope long peptide of MAGE-A4 cancer antigen was initiated.[104] A patient with pulmonary metastasis of CRC was vaccinated and had shown a significant reduction in the tumor growth.

NON-REACTIVE DISEASE—OVERCOMING THE UNDERLYING ISSUES

CTAs are key molecules in the field of cancer serology. Their progressively expanding family is continuously providing potential novel targets for cancer immunotherapy or diagnostic/prognostic examinations. However, CTAs have also been evaluated for their role in oncogenesis, particularly their contribution to the

immortality, invasiveness, immune evasion, hypomethylation and metastatic capacity of the neoplasms.[139] Investigating the correlation between CTAs and the clinical presentation of CRC (reactive or non-reactive type) requires knowledge of the molecular mechanisms that govern their expression and physiological functions—mechanisms that still remain poorly understood. According to recent studies, genome wide hypo-methylation has accounted for the aberrant expression of CTAs within CRC cells.[140–142] However, the epigenetic factors that dictate which panel of silenced genes to be reactivated and the physiological properties of these particular CTAs are still unknown. This is an area of actively ongoing research as it is believed that these factors are responsible for the heterogenicity in the CTA expression profiles of the individual CRC cases. A better understanding of the structural and serological properties of these antigens, their modes of expression and functions, should establish whether they are the ones governing the reactive and non-reactive CRC phenotypes.

CTAs in CRC have been associated with low immunogenicity under normal conditions, and only a few patients actually recognize the peptide epitopes and exhibit strong CTL or humoral immune responses to CTAs. This could be partially accounted to the structural stability of the proteins, encoded by the CT genes. It has been previously demonstrated that proteins must be cleaved to small peptides by intracellular proteinases prior to presentation to the immune system.[143] However, high stability proteins (a-helical secondary protein conformation known to provide the most optimal structural stability[141]) are less likely to undergo denaturation and polypeptide chain unfolding. Therefore, CTAs containing large proportions of a-secondary structure should be more resistant to protein unfolding and subsequent cleavage by the proteinases, thus resulting in an unsuccessful presentation to CTLs (low immunogenicity). For example, SCP-1 (a CTA of particularly low immunogenicity) has been shown to contain 76.6% a-helical structure from its total secondary conformation when compared with the highly immunogenic NY-ESO-1 CTA, containing only 20.7%.[141] The identification of such antigens that are predominantly expressed in non-reactive CRC patients could potentially be accounted for the therapy-resistant presentation of the disease. In such cases epigenetic modulation to induce expression of other CTAs may highly favor the immunotherapeutic approach to non-reactive CRC disease. In this respect, a study has recently reported a successful induction of NY-ESO-1 expression in CRC cells (but not in normal nontransformed cells) both in vitro and in vivo subject to hypomethylating agent 5-aza-2′-deoxycytidine (DAC) treatment.[144] The study reports that DAC-treated CRC cells are susceptible to MHC-restricted recognition by $CD8^+$ NY-ESO-1-specific T cells. A NY-ESO-$1_{157–165}$-specific T-cell receptor was successfully used to generate both $CD8^+$ and $CD4^+$ NY-ESO-$1_{157–165}$-specific T cells that selectively recognized DAC-treated CRC cells but not non-treated cells. These data reveal the great potential of combining epigenetic modulation and adoptive transfer of genetically engineered T lymphocytes targeting NY-ESO-1 or other CTAs for the development of a specific immunotherapy for CRC.

Another approach could also include the introduction of sequences capable of disrupting long a-helical stretches in the regions outside the potential epitopes.[141] Such approaches have the potential to improve the treatment response in patients with non-reactive type CRC. However, several challenges remain to be overcome, including the insufficient antitumour responses due to immunosuppression driven by T lymphocytes known as regulatory T cell (Tregs).

Infiltrating T lymphocytes

During the last decade, the search to understand the causes underlying presentation CRC has focused on Tregs. They have been shown to have a major role in cancer immunoevasion by directly inhibiting or even eradicating both CTLs and T-helper lymphocytes.[145] Tregs suppress autoreactive T cells without killing them through incompletely understood, contact-dependent mechanisms.[140] In healthy individuals, they represent 5-10% of the population of $CD4^+$ lymphocytes. However in cancer patients, Tregs may increase up to 30% and are predominantly found among the tumor-infiltrating lymphocytes present.[145] Therefore, the failure of the T and B cells to recognize and eradicate immunogenic cancer cells could be accounted for by the ability of certain tumors to secrete chemokine CCL22, which recruits Tregs and immobilizes the function of the anti-cancer immune response. Indeed patients with ulcerative colitis and irritable bowel syndrome have been shown to have higher numbers of infiltrating T cells than healthy controls[147] which may have a role in controlling cancer-driven inflammation. Tregs in CRC have been shown to exhibit both pro- and anti-tumor activities governed by the level of inflammatory stimuli received and dependent of the phase of tumourigenesis (early or late).[148] Following treatment in a Phase II clinical trial for CRC patients, the level of circulating Tregs was reported to have almost reached normal levels accompanied by 70% increase in CTLs responses against CEA epitopes.[149] Therefore, it still remains to be determined whether presence of Tregs in CRC has a pro- or anti- tumor role, how this correlation changes with the stage of the disease and the clinical significance of these changes.

In addition, Tregs appeared to be highly specific for a distinct set of TAAs in CRC patients, suggesting that Tregs exert T-cell suppression in an antigen-selective manner.[150] Several key hallmarks of Tregs in CRC have been identified, highlighting their complex role in the progression of the disease, the survival prognosis and their potential as therapeutic targets. Overall, it was established that CRC patients develop multivalent and individual T-cell responses against a broad variety of different CRC-associated TAAs. Therefore, selecting a panel of antigens according to pre-existing T-cell responses as an intermediary could improve the efficacy of future immunotherapies for CRC and should be further investigated. Better understanding of the role and behavior of Tregs within CRC would improve tumor profiling on an individual basis and could aid the choice of the most adequate therapy for each individual case. It could also benefit the development of cancer vaccines and other immune-based therapies targeting particular CTAs in non-reactive type CRC.

Tumor-infiltrating CD45RO(+)-cell density is a prognostic biomarker associated with longer survival in CRC patients, independent of clinical, pathological and molecular features.[151] Similar findings have also been reported from another study, linking the density of CD45RO(+) memory T cells in the different regions of CRC with dissemination to lymphovascular and perineural structures and to regional lymph nodes in patients— low densities were associated with a very poor prognosis.[152] However, not only was the type and density of the infiltrating immune cells within human CRC predictive of clinical outcome but also their location within the tumor, that is, whether they are located in the center of the tumor or the invasive margin. A strong in situ immune reaction in both regions was shown to correlate with a favorable prognosis regardless of the stage (I, II and III CRC).[153] Conversely, a weak in situ immune reaction predicted a poor clinical outcome even in patients with stage I CRC. These data were further supported by Page`s et al.[154] who examined the relationship between the presence of CD8(+) and CD45RO(+) in two regions of the tumor in patients with early stage CRC with regards to tumor recurrence and overall patient survival. High densities of both CD8(+) and CD45RO(+) correlated significantly with lower rates of CRC recurrence and increased overall survival when compared with patients with low densities of both immune cell types within the primary tumors. In contrast, extramural

vascular invasion and high FOXP3 ^{þ} cell density in lymphoid follicles were independent factors for worse survival, whereas a high frequency of lymphoid follicles in histologically normal colonic mucosa was associated with better survival.[155] Therefore, the collective analysis of the type, density and the location of immune cells within the CRC could be used to predict patient survival and to identify the high-risk patients who would benefit most from adjuvant therapy.

Intrinsically disordered proteins (IDPs)

Following a bioinformatics approach that implements the application of two algorithms (FoldIndex and RONN) predicting the level of disorder within a sequence, the experimental outcome revealed that 490% of the examined 228 CTAs were IDPs.[156] The latter are proteins that lack the typical hydrophobic cores and therefore do not appear as having rigid 3D structures (along their entire length or in localized regions) under physiological conditions and instead, exist as dynamic ensembles.[156,157] However, IDPs can evade being detected as 'misfolded' and degraded by the cell's surveillance system through their ability to undergo 'disorder-to-order' transitions upon binding to biological targets—a paradox known as the 'order/ disorder paradox'.[158] This is achieved as a segment of an IDP initially binds with the target, followed then by coalescing of the other protein segments facilitating the IDPs' folding.[159] However, recent studies have challenged this general view by revealing a phenomenon—uncoupled binding and folding of IDPs.[160] The complexity of the binding mechanisms of IDPs has been investigated by others[161,162] and a summary of possible pathways have been outlined in Figure 2.

The above mentioned properties associated with IDPs give an interesting angle of perception towards the expression, behavior and function of CTAs identified as IDPs. The lack of rigid 3D structures is believed to be responsible for the exposure of primary contact sites, which enables the faster, more effective and promiscuous binding at high concentrations to target mole-cules.[157] Together with the fact that intrinsic disorder has been identified as a determinant of genes that are harmful when overexpressed,[161] this could account for the correlation between CTA overexpression and disease prognosis. This is further supported by the fact that CTAs appear to occupy 'hub' positions (highly connected protein nodes) within the complex protein–protein interaction (PPI) network.[156,163] What was interesting in these findings was the fact that these networks are dynamic and grow incrementally by establishing new nodes. However, a desirable protein for recruitment to a hub position is a protein that is likely to participate in a large number of promiscuous interactions when overexpressed such as a CTA that is an IDP.[156] Eventually, the PPI network becomes dominated by such hubs leading to the overexpression of CTAs and to the creation of nodes with novel functions. This accounts for the poorer disease prognosis in later CRC stages and the phenotypic presentation of non-reactive CRC disease. This idea is further supported by the fact that a high abundance of IDPs is believed to result in undesirable interactions and potentially harmful effects of such interactions.[161] Perhaps the failure to respond to treatment in non-reactive type CRC could be due to targeting common nodes in a protein network rather than CTAs that occupy hub positions. Therefore, the search for such antigens could potentially take a turn towards more thorough investigation of the structure of the PPI networks established in cases of reactive disease.

CONCLUSIONS

Research over the last four decades has proven that CTAs hold a prominent role within the field of cancer serology and could be excellent diagnostic, prognostic and immunotherapeutic biomarkers in CRC. Furthermore, examination of their structural

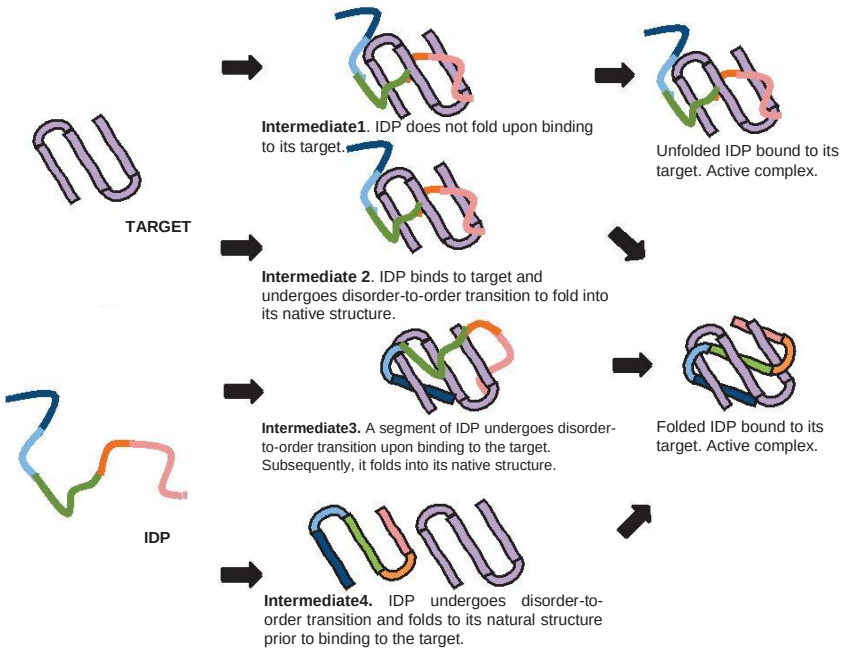

Figure 2. Diagrammatic representation of the different binding mechanisms and the disorder-to-order transition that the IDPs undergo before and upon binding to their targets (based on reference[160]). Some IDPs (particularly immune signalling-related IDPs) do not fold upon binding to their targets (for example, Intermediate 1). Other IDPs undergo partial or complete folding upon binding (Intermediate 2 and Intermediate 3) or fold before binding to their targets (Intermediate 4). The images shown are representative of the disorder-to-order transition that a hypothetical IDP would undergo.

properties and selective expression patterns may enable the understanding of the underlying molecular mechanisms that govern the reactive and non-reactive presentation of CRC. Nevertheless, the panel of CTAs with potential clinicopathological significance in relation to CRC still remains to be conclusively clarified. As this review pointed out, a large number of CTAs to date have been proven to be excellent prognostic biomarkers or targets for the development of peptide or protein vaccines in different solid tumors. However, the attempts to characterize such potent targets and to evaluate their significance in relation to CRC are still at their starting point. Up to date, the comprehensive analysis of the CTAs in relation to CRC have demonstrated that their secondary structural conformation and the presence of a large number of a-helical formations may be accounted for the reduced immunogenicity and treatment response of the CTAs associated with non-reactive type CRC. A solution to this potential problem has also been recently identified and evaluated—epigenetic modulation, which could induce the expression of highly immunogenic CTAs, that could subsequently be targeted by chemo- or immunotherapy. Particular attention should also be paid to the infiltration of Tregs within the tumor mass at different stages of the disease. Tregs have been associated with a rather complex and ambiguous role in the process of tumourigenesis in CRC patients as they have been demonstrated to exhibit both pro- and anti-tumor activities. Further study would be require to gain a better understanding on whether they play different roles in the presentation of reactive versus non-reactive CRC. Nevertheless, research on CTAs expression in CRC should follow the findings that these proteins are also exhibiting particular properties characteristic for IDPs.

In summary, the numerous studies and the significant data gathered regarding the expression pattern of CTAs and their role in carcinogenesis have not yet provided an explanation for the differences in the clinical presentation of CRC. However, they have demonstrated the complex variety of CTA expression mechanisms and their implications in hematological and solid cancers. The fact that certain CTA expressions could be indicative of poor prognosis in solid cancers but suggest good overall outcome in hematological malignancies brings to the fore a number of new challenges on the journey towards advancing clinical tumor immunotherapy. Still the main question remains unanswered: Can we transfer what triggers a good response in reactive disease to a patient with a poor anti-tumor response and improve the overall outcome? Identifying highly immunogenic peptides is a basic requirement for the development of immunotherapy vaccines. However, solid tumors, such as CRC pose additional obstacles on the road to mounting an immune response due to the fact that the antigens expressed within CRC cells are not readily accessible/easily recognized by the immune system. The heterogenicity in the nature of the tumor cells comprising CRCs and their multi-layered structure are the main reasons for evading immune recognition. However, understanding the underlying molecular basis of reactive disease could lead researchers to overcome these issues. Despite the amount of data gathered to date, the search for tumor-specific immunotherapy targets has remained elusive but as our understanding of how to induce effective immune responses grows so does our likelihood of improving patient outcomes.

FUTURE PERSPECTIVES

The identification of novel CTAs for CRC and evaluating the clinical significance of existing ones should be one of the main priorities in this field. As previous research has indicated, a number of CTAs could be effective prognostic biomarkers in CRC when evaluated as a panel of antigens rather than individually. The same approach could also be promising for the development of multivalent cancer vaccines that will target a panel of antigens in a particular tumor type.

It would also be beneficial to investigate the implications of high expression of novel CTAs on patient survival and treatment outcome. Furthermore, understanding the mechanisms of CTAs expression and epitope presentation to CTLs might provide new targets for epigenetic modulation or adjuvant therapies. Nevertheless, investigating the role of the known CRC-associated CTAs in relation to particular drug treatments or therapies should not be overlooked. As discussed earlier, a combined approach for the treatment of CRC might hold the key to the development of more successful therapies for CRC patients. Treatment with hypomethylating drugs on cancers with low CTA expression followed by immunotherapy has already shown promising results in both in vitro and in vivo studies. These findings should be further investigated in cases with CRC to provide a more conclusive idea of their effectiveness.

As suggested already, a more thorough investigation should enable the discovery of CTAs, which are also IDPs. Understanding the properties and the function of IDPs could assist a more comprehensive understanding of the physical and serological properties of CTAs and how they relate to CRC reactivity.

ABBREVIATIONS

AJCC, American Joint Committee on Cancer; AML, acute myeloid leukemia; CEA, carcinoembryonic antigen; CRC, colorectal cancer; CTA, cancer-testis antigen; CTL, cytotoxic T lymphocyte; DAC, 5-aza-2^0-deoxycytidine; DC, dendritic cells; EBV, Epstein–Barr virus; GIST, gastrointestinal stromal tumor; IDP, intrinsically disordered protein; pMHC, peptide MHC; RAYS, recombinant antigen expression on yeast surface; SADA, serum antibody detection array; SEREX, serological identification of antigens by recombinant expression cloning; SERPA, serological proteome analysis; SSX, synovial sarcoma X antigen; SSX2, synovial sarcoma X breakpoint-2; TAA, tumor-associated antigen; TNM, tumor nodes metastases; Tregs, regulatory T cells; TSP50: testes-specific protease 50.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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activity is known, lenalidomide has moderate CNS penetra-bility (Table I). In keeping with this, the patient had a com-plete neurological remission; pre-treatment neurological signs and symptoms resolved entirely and no plasma cells were detected either morphologically or by flow cytometry in the CSF. Despite the exquisite responsiveness in the CNS, the patient died with systemic disease 40 d later. Whilst it is not possible to determine the relative efficacy of dexamethasone or lenalidomide in this patient, we believe that the degree of CSF penetrability lends rationale to including lenalidomide as part of CNS-directed therapy for future patients.

In patients with relapsed or refractory myeloma, lenalido-mide can overcome resistance to conventional chemotherapy and has been shown to be effective in improving treatment outcomes both in terms of progression-free and overall sur-vival (Dimopoulos et al, 2007; Weber et al, 2007). No guide-lines for therapy or prevention of meningeal myelomatosis are available, which has resulted in large variation in treat-ment schedules. The therapeutic options include craniospinal irradiation, intrathecal chemotherapy, and systemic chemo-therapy, alone or in combination with each other. In this patient, progression occurred quickly after single agent bortezo-mib with dexamethasone. Increasingly, three- and four-drug combinations have been shown to be superior to conventional therapy in improving progression-free survival (Palumbo & Cavallo, 2012). It is to be hoped that these regimens would further reduce the risk of CNS disease.

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High and low, but not intermediate, PRAME expression levels are poor prognostic markers in myelodysplastic syndrome at disease presentation

The PReferentially expressed Antigen of the Melanoma Gene (*PRAME*) has been shown to be overexpressed in multiple solid cancers and in human germ-line tissues but only weakly expressed, if at all, in other healthy tissues leading to its clas-sification as a cancer-testis (CT) antigen. High expression of *PRAME* has been associated with poor survival and short-ened disease-free survival in a number of solid cancers. How-ever, evidence of an association between *PRAME* expression and survival in haematological malignancies has not been so clear. In acute promyelocytic leukaemia, typified by the t(15;17) translocation, low *PRAME* expression has indicated shortened overall survival (OS) (Santamaria et al, 2008), while high *PRAME* expression has also been associated with t(8;21), a favourable cytogenetic aberration, in acute myeloid leukaemia (AML) patients (van Baren *et al*, 1998). In addi-tion, *PRAME* expression was found to be associated with progression-free survival or OS in acute leukaemia by some groups (Greiner *et al*, 2006; Tajeddine *et al*, 2008) but not

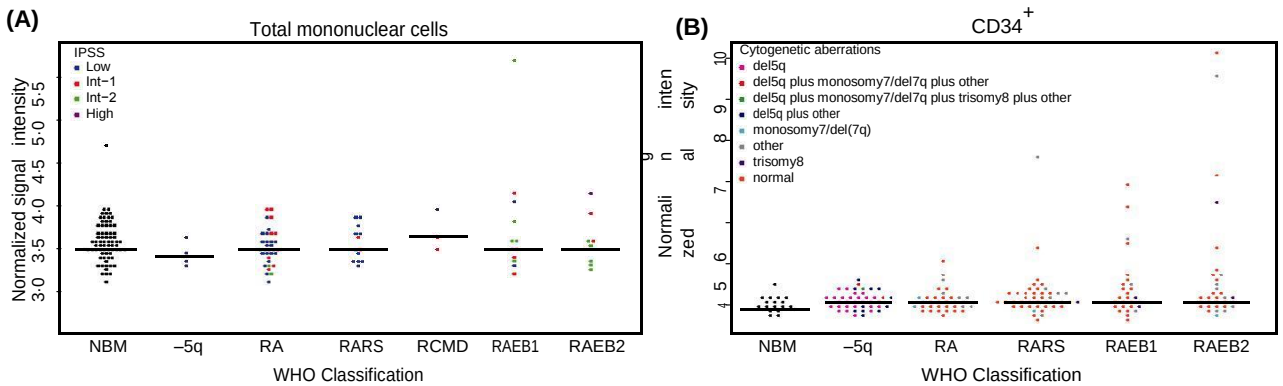

Fig 1. Normalized signal intensity of *PRAME*-targeted probe sets representing *PRAME* expression levels in (A) the mononuclear fraction of cells from 73 MDS patients. ANOVA analysis showed no difference in the relative *PRAME* expression across WHO groups. Bars represent the group median and data points were coloured based on IPSS category; (B) the $CD34^+$ fraction of cells from 183 MDS patients [and 17 healthy donors (NBM)] revealed that *PRAME* expression was statistically different across WHO sub-groups (P = 0 0152). Elevated *PRAME* levels were also found in $CD34^+$ cells from MDS patients with atypical cytogenetic aberrations when compared to those isolated from the NBM of healthy donors (P = 0 0489) (Figure 1B). Bars represent the group median and data points were coloured based on detected cytogenetic aberrations. ANOVA, analysis of variance; IPSS, International Prognostic Scoring System; WHO, World Health Organization; NBM, normal bone marrow; MDS, myelodys-plastic syndrome; RA, refractory anaemia; RA, refractory anaemia with ringed sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; RAEB1/2, refractory anaemia with excess blasts, type 1/2.

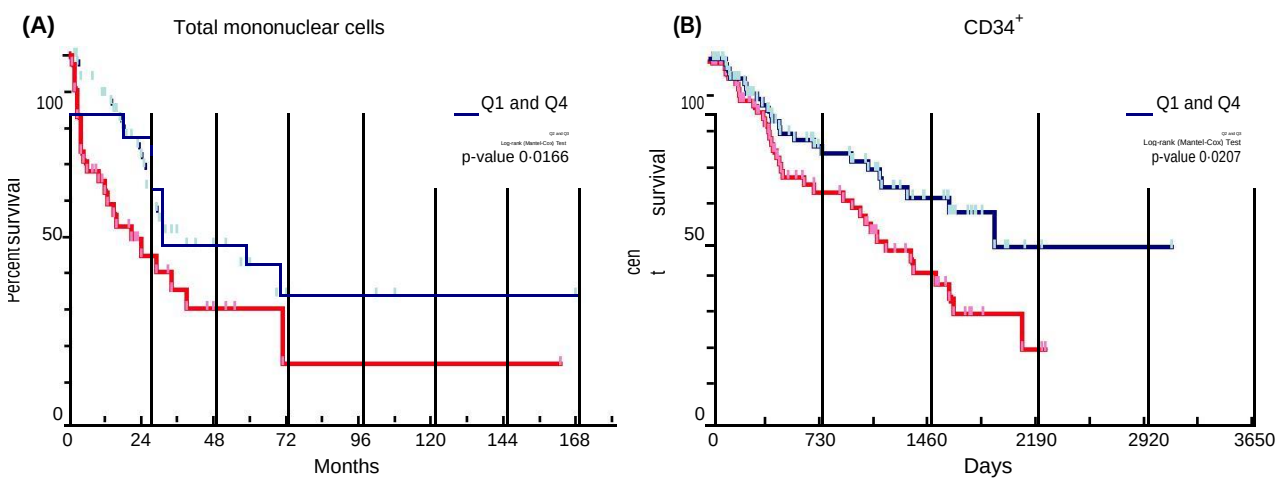

Fig 2. Kaplan–Meier survival curves based on relative PRAME expression in (A) the total mononuclear fraction (MNC, Data set A; n = 92 patients) and (B) the $CD34^+$ only fraction (Data set B; n = 166 patients for which follow-up data were available). Both cohorts of patients were stratified into two groups; those with a level of PRAME expression that lay within the interquartile range (n = 49 for MNC and n = 83 for $CD34^+$) and those with an expression level outside of this range (n = 43 for MNC and n = 83 for $CD34^+$); i.e. those with expression in the upper or lower quartiles. Each line represents patient survival according to these criteria. Patients with an intermediary expression of PRAME in their mononuclear cells or $CD34^+$ cells have the longest survival, whereas those with much higher or lower expression of PRAME have the shortest survival.

others (Paydas *et al*, 2005; Guinn *et al*, 2009). Evidence for the direct involvement of *PRAME* in carcinogenesis and disease progression was demonstrated in acute leukaemia cells when *PRAME* inhibition was shown to lead to apoptosis (Tanaka et al, 2011). However, other groups have reported that high expression of *PRAME* is a marker of a favourable outcome and good OS may be partly due to its association with a decreased expression of several other genes (*HSPB1*, *S100A4*, *CDKN1A*, *IL8* and *IGFBP2*) in childhood AML patients (Tajeddine *et al*, 2008).

Little evidence exists for the role of *PRAME* as a prognos-tic marker in myelodysplastic syndrome (MDS), the excep-tion being a recent report (Qian *et al*, 2011) showing that 11/56; 19 6% of MDS patients had *PRAME* gene promoter hypomethylation, a frequency that was higher in patients who were cytogenetically abnormal (5/21, 24%) compared with those who were cytogenetically normal (5/33, 15%). Qian *et al* (2011) also found that *PRAME* promoter hypome-thylation was elevated in MDS patients with subtypes associ-ated with more blasts [refractory anaemia with excess blasts

(RAEB) and RAEB in transformation] than those associated with less blasts [refractory anaemia (RA) and RA with ringed sideroblasts](37% versus 3%, P = 0 002). PRAME methylation status was identified as an indicator of OS (P = 0 026) with the 50% survival time for patients with hypomethylated PRAME being 11 months compared to 26 months for those with PRAME methylation. Qian et al (2011) performed multivariate analysis and showed that International Prognostic Scoring System (IPSS) classification (Low/Intermediate-1 versus Intermediate-2/High) and PRAME methylation status were independent prognostic factors (P = 0 009 and 0 005, respectively).

To further investigate whether PRAME levels at disease presentation could be used to indicate MDS patient survival we examined the mononuclear gene expression profiles (GEPs) of 92 presentation MDS patients (Data set A) (Mills et al, 2009) as well as a set of $CD34^+$ GEPs from 183 MDS patients and 17 healthy donors (Gene Expression Omnibus Accession number GSE19429) (Data set B) (Pellagatti et al, 2010). All samples were run on Affymetrix U133 Plus 2 microarrays and normalized using Robust Multiarray Averaging (RMA). Analysis of variance (ANOVA) of Data set A found no difference in the relative mononuclear levels of PRAME expression in MDS patients across the World Health Organization (WHO) groups (Fig 1A). However, analysis of expression profiles from the $CD34^+$ cells of Data set B revealed that PRAME expression was statistically different across WHO sub-groups (P = 0 0152). In addition, there were sig-nificantly higher levels of PRAME expression in MDS patients with at least three cytogenetic aberrations [including both 5q deletion (del(5q)) and chromosome 7 deletion (del (7)) or a deletion on 7q (del(7q))] when compared to $CD34^+$ cells from the normal bone marrow (NBM) of healthy donors (P = 0 0183). Elevated PRAME levels were also found in $CD34^+$ cells from MDS patients with atypical cytogenetic aberrations when compared to those from the NBM of healthy donors (P = 0 0489) (Fig 1B).

Kaplan–Meier analysis of MDS patients with PRAME expression above and below median levels showed there was no statistical difference between PRAME expression levels and OS (mononuclear cells P = 0 9744; $CD34^+$ P = 0 7781). However, when we used PRAME expression levels to divide the patients in Data set A into quartiles, we found that those with a PRAME expression level in the upper (Q1) or lower (Q4) quartiles showed poorer survival than those with an intermediate (Q2 and Q3) expression level. By combining patients into two groups, a significant reduction in OS was observed in patients with extreme PRAME expression levels (Q1 and 4) compared to those with PRAME expression within the interquartile range (Q2 and 3) (P-value of 0 0166 by Log-rank test) (Fig 2A). When patients in Data set B were similarly divided into two groups (Q1 and Q4 versus Q2 and Q3), in this case based on PRAME expression levels mea-sured in the $CD34^+$ compartment, a comparable reduction in OS was revealed (P-value of 0 0207 by Log-rank test) (Fig 2B). We performed a Fisher's exact test for independence, based on PRAME expression in the upper (Q1), inter-mediate (Q2 and Q3) and lower (Q4) quartiles versus the four IPSS groups on samples from Data set A. Although numbers were small, a P-value of 0 4944 indicated that PRAME is unlikely to be a surrogate for IPSS score, at least in this cohort.

Our data suggests a Goldilocks effect of the level of PRAME expression as a biomarker for survival. In this regard, either very high or very low levels of PRAME expression correlate with poor survival (Fig 2A,B). This apparent paradox may be explained by low levels of PRAME expression allowing leukaemia cells to evade the immune system, whilst higher levels of PRAME may reflect a higher tumour load and/or more aggressive leukaemia cells present at disease presentation. We believe that this is the first demonstration of a correlation between high and low PRAME expression levels in MDS and survival. The demon-stration of a Goldilocks effect, with regards to the level of PRAME expression and survival, may explain the variable interpretations of the impacts of PRAME levels on patient survival and should be considered when investigating other tumour-associated/CT antigens as potential biomarkers in the future.

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Author contributions

FL and AP performed the research, KIM and JB designed the research study, DTB provided essential reagents, FL, AP, KIM, JB and BG analysed the data and FL, KIM, VB and BG wrote the paper.

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VACCINATION AGAINST MYELOID LEUKAEMIAS USING NEWLY DEFINED ANTIGENS

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Running Head: New targets for leukaemia immunotherapy

Keywords: Tumour antigens, leukaemia, immunotherapy; clinical trials; myeloid leukaemia

Abbreviations: AML: acute myeloid leukaemia; CML: chronic myeloid leukaemia; CTA: cancer-testis antigen; CTL: cytotoxic T lymphocyte; DC: dendritic cell; DLI: donor leukocyte infusion; GvHD: graft-versus-host disease; GM-CSF: granulocyte-macrophage colony-stimulating factor; GvL: graft-versus-leukaemia; HSCT: haemopoietic stem cell transplantation; LAA: leukaemia-associated antigen; LSC: leukaemia stem cell; MAGE: melanoma associated antigen; MDS: myelodysplastic syndrome; MM: multiple myeloma; MRD: minimal residual disease; MUC1: mucin-1; NPM1: Nucleophosmin-1; PRAME: Preferentially expressed antigen of melanoma; RHAMM: receptor for hyaluronic-acid mediated motility;

SEREX: serological identification of antigens by recombinant expression cloning; SSX2IP: Synovial Sarcoma X breakpoint 2 interacting protein; WT1: Wilms' tumour antigen 1.

ABSTRACT

First complete remission rates are high in patients with acute myeloid leukaemia (AML) with some variation depending on the presence of specific cytogenetic and molecular aberrations. However the remission is often not long lasting and relapse occurs after standard chemotherapy within 2 years. Therefore besides chemotherapy, non-specific immunotherapy in the form of allogeneic haematopoietic stem cell transplantation is an integral part of consolidation and salvage therapy in the treatment of AML. A large number of leukaemia-associated antigens (LAAs) which can act as potential targets for specific immunotherapy have been identified and the number is still increasing. To date, several of these antigens are being utilised in clinical vaccination trials, either as active specific immunotherapy in form of peptide vaccination or as passive specific immunotherapy as adoptive cell therapies. In this review we will illustrate the role of newly defined LAAs as well as the results of already performed clinical vaccination trials with known LAAs to give an overview and evaluation of the state-of-the-art.

INTRODUCTION

Allogeneic haematopoietic stem cell transplantation (HSCT) and donor leukocyte infusion (DLI) with its crucial graft-versus-leukaemia (GvL)-effect demonstrate the important role of immunotherapy in the treatment of leukaemia and other malignant haematologic diseases (Figure 1). The GvL-effect is based on a predominantly cytotoxic T lympphocyte (CTL) mediated immune response which targets minor histocompatibility antigens (mHAgs) (Kloosterboer et al.,

2004) and leukaemia-associated antigens (LAAs). The use of unselected immunotherapy in the form of HSCT and DLI can initiate graft-versus-host disease (GvHD) with devastating consequences indicating the need to develop more leukaemia-specific immunotherapies. At this point, LAAs which show an expression that is almost specific to leukaemia cells and more importantly to leukaemic stem cells (LSC), come into play. These act as gate keepers to CTLs which are able to be **exquisitely specific** in their elimination of malignant cells. However due to the low frequency of LAA-specific CTLs, studies have been inaugurated to expand LAA-specific T-cells in vitro for passive immunotherapy as a form of adoptive T-cell transfer or in vivo for active immunotherapy with the help of peptide vaccination to induce or potentiate LAA-specific T-cell responses.

LAAs

The first tumour-associated antigen, called melanoma associated antigen (MAGE), was identified in the early 1990s by T-cell expression cloning. The subsequent development of the serological identification of antigens by recombinant expression cloning (SEREX) technique has allowed the rapid identification of antigens from a range of tumour types. A repository detailing more than 2,000 antigens found by this technique has been developed and called the *cancer immunome database* (http://ludwig-sun5.unil.ch/CancerImmunomeDB/).

To prioritize the myriad of identified antigens, Cheever et al. (2009) devised a list of criteria to define the “ideal” target antigen with the order representing the weight of each characteristics

(Table 1). Although there is no antigen that fulfills all of the criteria some LAAs seem to be more suited for use in clinical vaccination than others.

We focus our review on a short list of LAAs which best fit the criteria. All of these antigens bear epitopes that are recognized by $CD8^+$ T cells (CTLs) and for some of them humoral immune responses have been described as well (Greiner et al., 2006). The importance of immune mechanisms in the success of leukaemia immunotherapy is strengthened by a study using microarray analysis in which an association between elevated LAA expression and survival in AML patients was found. Expression of at least one of three LAAs - receptor for hyaluronic-acid mediated motility (RHAMM), preferentially expressed antigen of melanoma (PRAME), or G250 – had a favourable prognostic score ($p = 0.005$)(Greiner et al., 2006). Similar results were also found for the coexpression of three LAAs Synovial Sarcoma X breakpoint 2 interacting protein (SSX2IP), Survivin or RHAMM in AML ($p = 0.0071$)(Guinn et al., 2009). Together with the knowledge that several LAA-specific CTLs are skilled to lyse autologous leukaemic blasts (Molldrem et al., 1996, Gao et al., 2000), these data led to the hypothesis that the coexpression of distinct LAAs on leukaemic blasts facilitates their eradication by eliciting T-cell responses following conventional therapy, in a subgroup of patients who respond well to treatment. We hypothesised that cell lysis and inflammation around the site of the tumour in these patients causes the appropriate “danger” signals to elicit a longer lasting remission due to the effective induction of the immune response to kill surviving tumour cells.

LAA EXPRESSION IN LSC

One reason for the limited and often short-term success of conventional chemotherapy in the treatment of AML is that the agents used are not able to eliminate chemoresistent or quiescent LSC. Efforts have been made to uncover this subpopulation through their markers to allow

minimal residual disease (MRD) monitoring by real-time PCR. For AML patients with a nucleophosmin-1 (*NPM1*)-mutation in their blasts it is the *NPM1*-mutation load which is a prognostic marker for relapse of the disease. Wilms' tumour antigen 1 (WT1) represents another molecular marker for early assessment of MRD as the expression levels in normal haematopoietic progenitor cells are at least ten times less than those in leukaemic cells (Inoue et al., 1997). Therefore, it is of particular interest to develop therapies to eradicate MRD and several working groups have performed gene expression profiling on LSCs to characterize the differences between leukaemic and normal HSCs. LAAs that are expressed by LSCs are of special interest as they form the contact point for LSC killing by CTLs and therefore for LSC-specific immunotherapy.

PROMISING RESULTS FOR VACCINATION TRIALS WITH KNOWN LAAS

PRAME

PRAME was identified as a human melanoma associated antigen recognized by autologous CTL although its function still remains unclear. PRAME has been shown to be overexpressed in multiple solid cancers and in human germ-line tissues but not or only weakly expressed in other healthy tissues. Therefore, PRAME is classified as a cancer-testis antigen (CTA). However, unlike most of the other CTAs, PRAME expression has been demonstrated in several haematological malignancies including AML and chronic myeloid leukaemia (CML).

A growing body of evidence now indicates that PRAME antigen is an effective MRD marker for leukaemia. This could permit prognostic evaluation of each patient shortly after treatment. Interestingly, independent studies on adult AML (Zhou et al., 2007, Greiner et al., 2006) have

reported that high expression of PRAME is a marker of a favourable outcome and good overall survival. This has been accounted for, at least in part, by the finding that PRAME alters the expression of several genes (Hsp27, S100A4, p21, IL-8 and IGFBP-2) involved in carcinogenesis, and cancer progression in the case of childhood AML (Tajeddine et al., 2008). Similar findings have been reported in cases of acute promyelocytic leukaemia where low PRAME expression has indicated shortened relapse-free and overall survival when compared to patients with high PRAME expression (Santamaria et al., 2008). However, another study suggested that PRAME was directly involved in carcinogenesis and disease progression and its inhibition led to the apoptosis of leukaemic cells (Tanaka et al., 2011).

Although PRAME has been targeted in a number of clinical trials for solid tumours in published and ongoing clinical trials, in myeloid leukaemia the main focus has been the demonstration of PRAME responses post-treatment. This indicates the tumour-specific targetting of CTLs (Li et al., 2006) reflecting the immunogenicity of PRAME in the appropriate context.

RHAMM

RHAMM is an extracellular matrix protein involved in cell differentiation, proliferation and motility. It was shown to be immunogenic and to induce humoral and cellular immune responses (Greiner et al., 2005). mRNA of RHAMM was found to be expressed in AML patients in contrast to normal tissues and strong expression of RHAMM mRNA was only found in the testis, placenta and thymus.

A clinical phase I/II peptide vaccination trial with RHAMM-R3 involving two cohorts of patients overexpressing RHAMM has been performed. The HLA-A2- restricted RHAMM-R3 peptide was emulsified in incomplete Freund's adjuvant and as a second adjuvant, granulocyte-macrophage colony-stimulating factor (GM-CSF) was administered on day 0 until day +2. The patients were

vaccinated four times subcutaneously and in a biweekly interval. Altogether, the trial showed no drug-induced adverse events higher than the common toxicity criteria (CTC) grade 1 skin category. The first 10 patients were vaccinated with 300µg of the peptide and the following 9 patients with 1000 µg of the peptide (Greiner et al., 2010). The first cohort showed specific immune responses in 70% of the patients and positive clinical effects in 50% of the patients with a reduction of blasts in the bone marrow in patients with myeloid disorders or a decrease in free light chain serum levels in patients with multiple myeloma (MM). In one patient with myelodysplastic syndrome (MDS), erythrocyte transfusions were no longer necessary.

In the second cohort with the higher RHAMM-R3-peptide dose, 44% of vaccinated patients showed an increase in RHAMM-R3-specific T cells in tetramer and ELISpot assays as surrogate parameters for the immunogenic effects of the vaccine. A decrease in specific T-cell responses could be observed in some patients after vaccination was stopped, maybe indicating a failure to induce high-avidity T–cell activity, or memory responses, against the LAA.

WT1

WT1 has emerged as one of the most promising targets for AML immunotherapy while also being applicable to the treatment of other haematological and solid tumours. Despite its ubiquitous expression during embryogenesis, WT1 expression in normal individuals is limited to renal podocytes, gonadal cells and a small proportion of normal $CD34^+$ cells where expression is significantly lower (10-100 fold). The WT1 gene was originally defined as a tumour suppressor gene, however, recent studies suggest that in adults with solid and haematopoietic malignancies it also exhibits an oncogenic function. WT1 is often overexpressed and detected in tumour-bearing patients where it can induce specific CTL and antibody responses (Li et al., 2008). In addition blocking of WT1 expression has been shown to significantly slow the growth of

leukaemic blast cells (Yamagami et al., 1996). These properties make WT1 an attractive target antigen for developing WT1-based adoptive T cell therapy and vaccination strategies against various types of cancers.

WT1 peptide vaccines have been studied *in vitro* and in clinical trials (Rezvani et al., 2008) showing some immune responses but poor persistence. Three peptide vaccines, each encoding a different previously described WT1-derived, HLA-A2-restricted epitope, were tested in "humanised" mice expressing a chimeric HLA-A2 molecule (Chaise et al., 2008) and induced killing of human $WT1^{+}HHD^{+}$ leukaemic cells *ex vivo*.

Oka et al*.,* (2004) performed a phase I clinical trial of a WT1-derived vaccine involving patients with a variety of solid and haematopoietic malignancies. In 2004 the outcome of the trial had demonstrated the safety of the vaccine and their capacity to expand WT1-specific CTLs. The first phase II trial for WT1 peptide vaccination was conducted on patients with AML and MDS whose blasts expressed WT1 (Keilholz et al., 2009). The aim of the study was to investigate the safety and efficacy of a combination of GM-CSF (days 1-4) as an immune-enhancement adjuvant along with the WT1.126-134 peptide and keyhole limpethemocyanin vaccine (on day 3). The vaccination was reported to have been well tolerated and to have led to blast reduction and overall increase in WT1-$tetramer^{+}$ T cells in the blood and bone marrow of AML patients, suggesting its clinical efficacy in AML patients. Further details of recruiting or ongoing phase I and II clinical trials targeting WT1 can be found in Table 2.

NEW TARGETS FOR IMMUNOTHERAPY

Aurora kinase A and B

Aurora kinases are a family of serine/threonine protein kinases (Auroras A, B and C) which play an important role in chromosomal seggregation and cell division (Meraldi et al., 2004). Aurora kinase A and B have both been shown to be elevated in AML patient samples. Aurora kinase inhibitors, in the form of small molecule therapies, have entered Phase I clinical trials for solid tumours (Arkenau et al., 2012) while specific antibodies have been used in Phase I/II clinical trials on AML patients (Dennis et al., 2012). Both have been shown to be safe, tolerable and to have some preliminary efficacy. *In vitro* such inhibitors have been shown to be able to induce growth arrest in a BCR-ABL independent manner and to circumvent imatinib resistance in BCR-ABL^+ cells (Mancini et al., 2012). Chemotherapy appears to potentiate the effect of aurora kinase inhibitors in AML (Yoshida et al., 2011). Recently, epitopes derived from Aurora kinase A and B have been shown to elicit $CD8^+$ T cell responses in patients with AML (Schneider et al., 2012) which may provide an alternative way of killing leukaemic blast cells with upregulated Aurora kinase levels.

G250

G250/CA IX is a membrane associated protein and part of the carbonic anhydrase family. It is described as a marker of hypoxia and is regulated by hypoxia-inducible factor alpha. The LAA G250/CA IX is expressed in variant solid malignancies but also AML, whereas no expression could be detected in normal cells. No clinical trials have been initiated targetting G250 in AML although there have been for other solid tumours, particularly renal cell carcinoma (reviewed most recently in (Santoni et al., 2012)).

HAGE

HAGE is a member of the DEAD-box family of ATP-dependent RNA helicases. Other members are involved in tumour cell proliferation suggesting a role for HAGE in the RNA metabolism and/or control of cell cycle within tumour cells. Like most CTAs, HAGE expression correlates with hypomethylation of the promoter region and increased expression has been associated with disease progression (Adams et al, 2002; Chen et al., 2011). HAGE expression has been demonstrated in CML patients (Adams et al., 2002, Chen et al., 2011). Recent studies indicated that the knockdown of HAGE expression by small interfering RNA (siRNA) led to a significant decrease in Neuroblastoma RAS protein expression with a concomitant decrease in activation of the AKT and ERK signaling pathways in malignant melanoma-initiating cells which are thought to play a central role in melanoma progression (Linley et al., 2012). In addition Linley et al showed a significant decrease in the tumour growth of $ABCB5^{+}$ melanoma cells in non-obese diabetic/severe combined immunodeficiency mice. This demonstrates that therapies targeting HAGE helicase may have direct effects on all cancer cells in which HAGE plays a role.

MUC1

The highly glycosylated type I transmembrane glycoprotein mucin-1 (MUC1) is overexpressed in many human adenocarcinomas and it is expressed in MM and some B-cell lymphomas. Brossart et al. found MUC1 expression in 67% of AML blast cells, especially FAB M4 and M5 subtypes (Brossart et al., 2001). In addition the group showed that MUC1 specific CTLs could be induced by peptide pulsed dendritic cells (DCs) from healthy donors, and that these could lyse HLA-A2 positive MUC1 positive tumour cell lines and primary AML blasts. MUC1 has been shown to stabilize the BCR-ABL oncoprotein typical of CML cells and to play a role in the pathogenesis of

the disease by promoting CML blast cell self renewal while inhibiting differentiation and apoptosis (Kawano et al., 2007). Silencing MUC1 has also been shown to increase CML cell sensitivity to imatinib by the same group.

NPM1

The mutated NPM1 ($NPM1^{mut}$) protein plays a special role in AML, as it is a prognostic marker and AML with $NPM1^{mut}$ belongs to the favorable group of the ELN classification. Mutations in the *NPM1* gene are one of the most frequent single gene mutations in AML (25%-30%) and they predominantly occur in AML with normal karyotype (45%-60%) (Schlenk et al., 2008). Several mutations of the *NPM1* gene exist in AML, but more than 90% harbour the so-called A, B and D mutations (Falini et al., 2007). *NPM1* mutation dislocates the nuclear expressed wild-type protein into the cytoplasm and hence interferes with the ARF/p53 pathway and NF-κB.

The favourable prognosis of AML with $NPM1^{mut}$ led to the hypothesis that immune responses may contribute to the positive outcome by specific lysis of residual leukaemic cells that bear the $NPM1^{mut}$. Therefore, epitopes derived from the mutated regions of *NPM1* were analyzed with regards to their ability to induce specific $CD4^+$ and $CD8^+$ T-cell responses (Greiner et al., 2012). Two HLA-A2 restricted epitopes, induced high frequencies of specific $CD8^+$ T-cell responses in healthy volunteers and AML patients (Hofmann et al., 2013).

In AML with $NPM1^{mut}$, the $NPM1^{mut}$ load can be used to detect MRD by real-time quantitative polymerase chain reaction and more than 200 $NPM1^{mut}/10^4$ ABL copies or an increasing load over time indicates early relapse in the post-treatment phase (Kronke et al., 2011). Patients harbouring a low tumour burden represented by MRD without haematological relapse, could be

vaccinated to expand *NPM1*^{mut}–specific CD8^{+} T-cells to prolong remission or even cure the disease.

PASD1

PASD1 was identified as a CTA in diffuse large B-cell lymphoma (Liggins et al., 2004) and AML (Guinn et al., 2005). The gene maps to chromosome Xq28 and alternative splicing produces at least two variants, *PASD1_v1* and *PASD1_v2* (Liggins et al., 2004). PASD1 may be an important CTA for haematological malignancies as it is frequently expressed in myeloid leukaemia (Guinn et al., 2005). There is evidence that CD4 and CD8 T cells can respond to PASD1 epitopes in patients with lymphoma (Ait-Tahar et al., 2009) and myeloid leukaemia (Hardwick et al., in press) while DNA vaccines targeting PASD1 epitopes have shown efficacy in HLA-A2 chimeric HHD mice (Joseph-Pietras et al., 2010)(Hardwick et al., in press).

SSX2/SSX2IP

The SSX family of genes are CTAs which include SSX2. SSX2 is expressed in a range of leukaemia cell lines (Figure 2) as well as 50% of human melanomas, 25% of colon cancers, 30% of hepatocarcinomas and 20% of breast carcinomas (Tureci et al., 1996). Synovial sarcomas are characterised by the t(X; 18) translocation which sees the fusion of SSX2 with the gene SYT on chromosome 18. SSX2 has been found to have a strong association with reduced survival in MM ($p = 0.0001$) (Taylor et al., 2005) and overexpression of SSX2 induces cell growth and prompts cell invasion by suppression of ERα and E-cadherin (Chen et al., 2012).

SSX2 interacting protein (SSX2IP) was identified by a yeast two-hybrid screening system as an associate of SSX2 (de Bruijn et al., 2002). Elevated expression of SSX2IP at disease presentation was associated with improved survival (Guinn et al., 2009) in AML patients who lacked cytogenetic abnormalities while levels of SSX2IP expression in AML patients harboring a t(8;21) translocation were reduced (Guinn et al., 2007). Using microarray datasets, associations between SSX2IP and the genes involved in spindle checkpoints were found. A strong correlation between low-CDC20 expression, one of the substrate-targeting subunits of the anaphase-promoting complex and low SSX2IP expression in patients harbouring a t(8;21) translocation ($P<0.0001$) suggested that SSX2IP may also play a role in mitotic spindle failure in this group of patients. Indeed SSX2IP expression was increased in patients who have the t(15;17) translocation and found to be significantly associated with genes involved in the cell cycle.

SUMMARY

Results from completed clinical phase I/II trials in which LAAs in the form of peptides or administered via DC vaccination strategies are encouraging. Although none of the LAAs listed above fulfil all criteria for an ideal target antigen, clinical efficacy as well as immunogenicity can be influenced by the optimal regimen. Careful consideration of vaccination intervals, use and choice of an adjuvant and dosage remain to be optimised in many studies. In the post-treatment/remission phase, when a low tumour burden is achieved through chemotherapy and/or allogeneic HSCT, immunotherapy appears to be a promising tool to remove residual disease and support a longer-lasting remission of the leukaemia with the potential to lead to cures.

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Table 1. Characteristics of the "ideal" target antigen as applied to myeloid leukaemias (based on criteria developed by Cheever et al. 2009)

Characteristics of the "ideal" target antigen	**Examples of antigens fitting this criteria**	**References**
(a) Induce clinical effects	WT1	Rezvani et al., 2008
		Oka et al., 2004
		Keilholz et al., 2009
	RHAMM	Greiner et al., 2010
	G250	Santoni et al., 2012
	Aurora kinase A & B	Arkenau et al., 2012
		Dennis et al., 2012
	NPM1 mutation	Hofmann et al., 2013
(b) Immunogenic	PRAME	Li et al., 2006
	RHAMM	Greiner et al., 2005
	WT1	Chaise et al., 2008
	MUC1	Brossart et al., 2001
	PASD1	Ait-Tahar et al., 2009
		Joseph-Petras et al., 2010
		Hardwick et al., in press
(c) Critical role in cell differentiation and proliferation of the malignant cells or characterize the malignant cell	PRAME	Tanaka et al., 2011
	WT1	Yamagami et al., 1996
	Aurora kinase A & B	Mancini et al., 2012
	HAGE	Linley et al., 2012
	MUC1	Kawano et al., 2007
	SSX2IP	Guinn et al., 2007
(d) Expression restricted to malignant cells	G250	Greiner et al., 2006

(e) Expression in all malignant cells		
(f) Expression in malignant stem cells	NPM1 mutation WT1	Falini et al., 2007 Keiholz et al., 2009
(g) High number of patients bearing antigen-positive cells	WT1 NPM1 mutation PASD1 SSX2IP	Inoue et al, 1997 Falini et al., 2007 Guinn et al., 2005 Guinn et al., 2005
(h) Includes multiple antigenic epitopes	WT1 Aurora kinase A & B NPM1 mutation PASD1	Chaise et al., 2008 Schneider et al., 2012 Greiner et al., 2012 Hofmann et al., 2013 Ait-Tahar et al., 2009 Hardwick et al., in press
(i) Expressed on the cell surface	SSX2IP	Denniss et al., 2007

Table 2. Active Phase I and II clinical trials targeting WT1 in the myeloid leukaemias

No.	Phase	Purpose	Cancer type	Principal Investigator	Status	Date to be completed:	Identifier (ClinicalTrials .gov)
1	I, II	To evaluate the safety and the efficacy of combined treatment strategy: Recombinant WT1 -A10 + AS01B Antigen-Specific Cancer Immunotherapeutic (ASCI) combined with regulatory T cell depletion.	WT1-positive AML	Institute Jules Bordet	Active	12/2014	NCT01513109
2	I	To evaluate the safety and efficacy of treatment with WT1-sensitized T cells.	WT1-positive residual or relapsed leukaemia after allogeneic hematopoietic progenitor cell transplantation.	R. O'Reilly	Active	02/2013	NCT00620633
3	II	To evaluate the safety and efficacy of treatment with p.DOM-epitope DNA vaccination in patients with WT1-positive CML and AML.	WT1-positive CML, ALL, APL and AML	K. Rezvani	Active	08/2012	NCT01334060
4	I	To evaluate the safety and efficacy of treatment with WT1 peptide vaccine in combination with Montanide ISA-51 and GM-CSF.	WT1-positive AML, CML, ALL, MDS, B cell malignancies.	M. Morse	Active	06/2014	NCT00672152
5	II	To evaluate the safety and efficacy of GSK2302024A antigen-specific cancer immunotherapeutic combined With Standard Neoadjuvant Treatment.	WT1-positive primary invasive breast cancer	Pharmacy/ Industry	Active	12/2013	NCT01220128
6	II	To evaluate the safety, immunogenicity and antitumour activity of WT2725.	WT1-positive advanced solid malignancies	Pharmacy/ Industry	Active	09/2013	NCT01621542
7	I, II	To determine the safety, effectiveness, immune response and side effects of giving WT1 peptide-pulsed DCs and donor white blood cells to patients.	WT1-positive AML, ALL, CML,MDS, NHL	A. Wayne	Active	11/2015	NCT00923910
8	I, II	To evaluate the safety and efficacy of WT1 TCR-transduced T cells.	WT1-positive AML and CML	E. Morris	Active	04/2016	NCT01621724
9	II	To evaluate the safety and efficacy of WT1-vaccine Montanide + GM-CSF	WT1-positive malignant pleural mesothelioma	L. Krug	Active	12/2014	NCT01265433

Figure 1: Schematic presentation of the need of immunotherapy in the treatment of AML. At the time of diagnosis, the load of leukemic blasts (yellow circles) as well as of leukaemic stem cells (red circles, MRD minimal residual disease as detected by WT1 and/or $NPM1^{mut}$ expression) is high and minimises the space available to normal haematopoietic cells (blue circles) in the bone marrow. After chemotherapy cycles, the MRD burden decreases as well as the burden of leukaemic blasts but often quiescent or chemoresistant leukaemic stem cells are not eliminated. Immunotherapeutic approaches have the potential to target LAAs expressed in and on leukaemic stem cells such that a LAA-specific vaccine could boost LAA-specific cytotoxic T-cells and lead to a long-lasting remission.

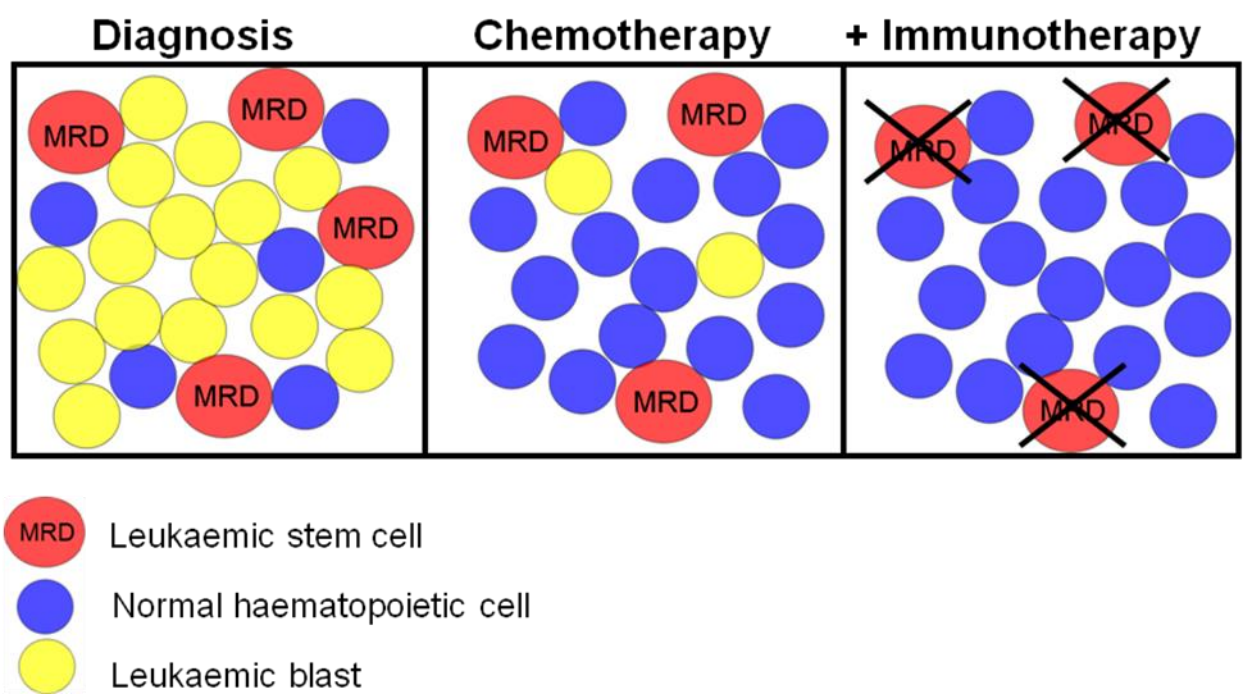
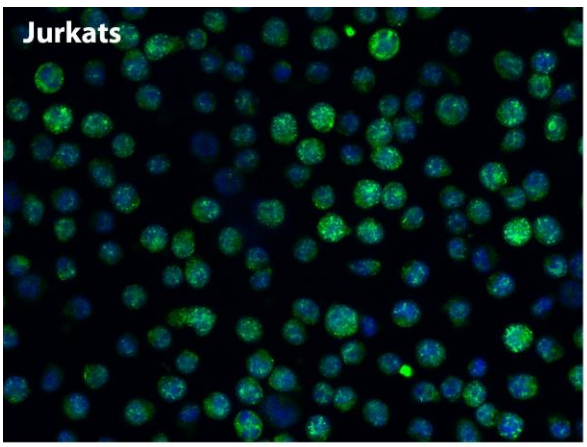
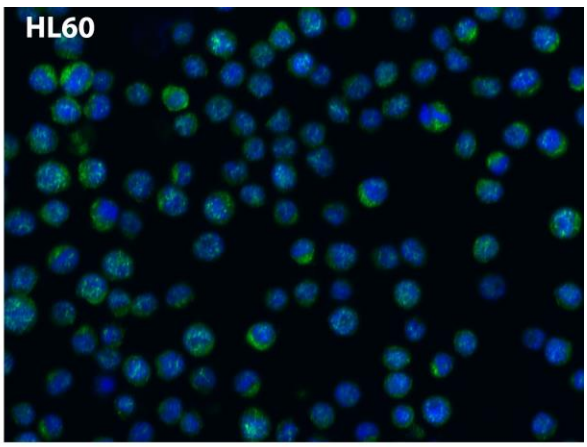
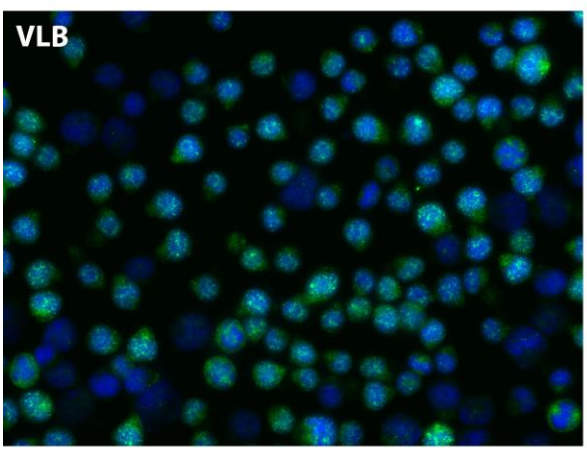
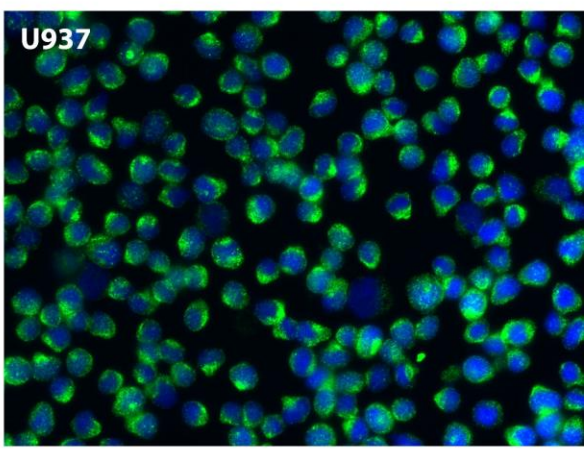


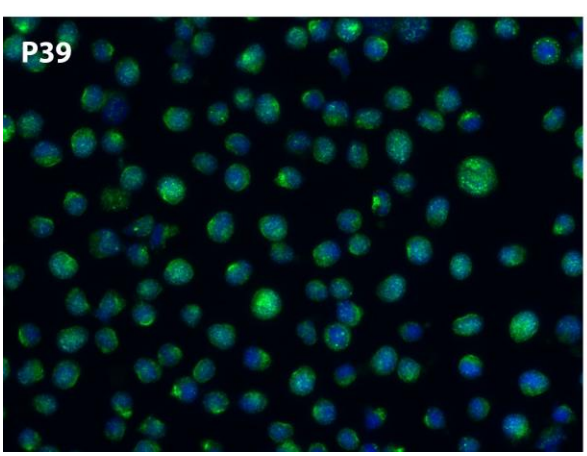
Figure 2. SSX2 expression in human leukaemia cell lines. Leukaemia cell lines were spotted at 5 x 10^6 cells per ml in PBS and air dried. Cells were fixed in cold methanol, permeabilised with 0.25% saponin 20min (with 0.05% saponin in all subsequent buffers). Slides were washed 3 x in TBS and then incubated with 40µl of primary mouse rabbit anti-human SSX2 antibody (clone 4d10) from Abcam (1/200) was incubated with the cells overnight at 4^{o}C. Slides were washed 3 x in TBS and then incubated with 40µl of goat anti-rabbit:AF488, diluted 1/1000 (Life Technologies) was incubated with the cells for 1hr and visualised following the addition of Vectamount with DAPI (Vector Labs). Nuclei were stained with DAPI (blue). Images were collected with an Olympus CKX inverted microscope with reflected fluorescence system with Olympus CellB software. Blue and green images (TIFF) were transferred to Adobe Photoshop CS5 merged and contrast stretched to use the whole grey scale. Jurkats is a human T cell leukaemia cell line, the white bar shows a 100µm scale bar) while HL60, VLB, U937, P39 and NB4 are myeloid leukaemia cell lines. Intracellular staining with SSX2 is predominantly nuclear with some cytoplasmic except U937 (mostly cytoplasmic) and NB4 (strong nuclear and cytoplasmic staining).

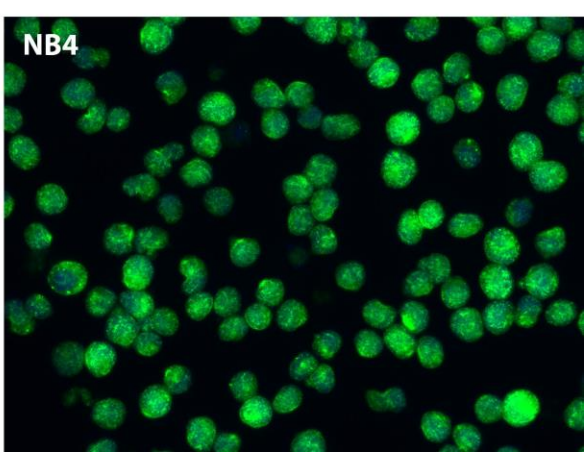

Jurkats


HL60


VLB


U937


P39


NB4

Appendix II SEREX Primary Immunoscreening

Date of experiment and Plate No.	SERUM ID	Plaques cored out and label	Total No. of plaques	Plaques confirmation - date of Secondary SEREX screening	Date of isolating positive plaques	No. Of isolated plaques
29.1.2013 1	CC005 1:2000	310113-1-1 310113-1-2	4116	negative (22/5/13) (CC005) negative (22/5/13) (CC005)	/ /	/ /
29.1.2013 2	CC005 1:2000	0	3716	/	/	/
29.1.2013 3	CC005 1:2000	0	3948	/	/	/
29.1.2013 4	CC005 1:2000	310113-4-1	4452			
29.1.2013 5	CC005 1:2000	310113-5-1 310113-5-2	3516	negative (22/5/13) (CC005) negative (22/5/13) (CC005)	/ /	/ /
29.1.2013 6	CC005 1:2000	0	3736	/	/	/
29.1.2013 7	CC005 1:2000	310113-7-1	3656	negative (22/5/13) (CC005)	/	/
29.1.2013 8	CC005 1:2000	0	3452	/	/	/
06.2.2013 1	CC014 1:200	060213-1-1	3924			
06.2.2013 2	CC014 1:200	060213-2-1	3072	UOB-COL-1 (19/02/13)	20/02/13 21/06/13	3x(CC005) 3x(CC014) negative with CC014 negative with CC005 negative with CC010
		060213-2-2		negative (27/06/13) (CC005) negative (27/06/13) (CC010) negative (27/06/13) (CC014) negative (13/07/13) (CC014)	/	/
06.2.2013 3	CC014 1:200	060213-3-1	3248	negative (27/06/13) (CC005) negative (27/06/13) (CC010) negative (27/06/13) (CC014) negative (13/07/13) (CC014)	/	/
		060213-3-2		negative (27/06/13) (CC014)		
06.2.2013 4	CC014 1:200	0	3724	/	/	/
06.2.2013 5	CC014 1:200	0	4124	/	/	/
06.2.2013 6	CC014 1:200	060213-6-1	2916			
12.2.2013 1	CC014 1:200	0	3452	/	/	/

12.2.2013 2	**CC014 1:200**	130213-2-1	**3140**			
12.2.2013 3	**CC014 1:200**	130213-3-1	**3332**			
		130213-3-2		**negative (07/03/13) (CC014)**	**/**	**/**
12.2.2013 4	**CC014 1:200**	130213-4-1	**3656**	**negative (21/06/13) (CC005)** **negative (21/06/13) (CC010)** **negative (21/06/13) (CC014)**	**/**	**/**
		130213-4-2		**negative (28/02/13) (CC014)**	**/**	**/**
		130213-4-3				
		130213-4-4				
		130213-4-5		**negative (07/03/13) (CC014)**	**/**	**/**
		130213-4-6				
12.2.2013 5	**CC014 1:200**	130213-5-1	**3852**	**negative (07/03/13) (CC014)**	**/**	**/**
18.2.2013 1	**CC005 1:200**	0	**TMTC**	**/**	**/**	**/**
18.2.2013 2	**CC005 1:200**	190213-2-1	**4300**			
		190213-2-2				
		190213-2-3				
		190213-2-4				
18.2.2013 3	**CC005 1:200**	190213-3-1	**3956**	**negative (21/03/13) (CC005)**	**/**	**/**
				UOB-COL-8 (CC005) (7/4/13)	**07/04/13**	**8x(CC005)**
18.2.2013 4	**CC005 1:200**	190213-4-1	**4844**			
18.2.2013 5	**CC014 1:200**	190213-5-1	**4128**	**UOB-COL-2 (28/02/13)**	**07/03/13**	**3x(CC014)**
18.2.2013 6	**CC014 1:200**	190213-6-1	**4404**	**negative (07/03/13) (CC014)**	**/**	**/**
		190213-6-2				
26.2.2013 1	**CC014 1:200**	260213-1-1	**4256**			
26.2.2013 2	**CC014 1:200**	0	**4276**	**/**	**/**	**/**
26.2.2013 3	**CC014 1:200**	260213-3-1	**4152**	**negative (21/03/13) (CC014)**	**/**	**/**
		260213-3-2		**negative (06/07/13) (CC005)** **negative (06/07/13) (CC010)** **negative (06/07/13) (CC014)** **negative (13/07/13) (CC014)**	**/**	**/**
		260213-3-3		**negative (27/06/13) (CC005)** **negative (27/06/13) (CC010)** **negative (27/06/13) (CC014)** **negative (13/07/13) (CC014)**	**/**	**/**
26.2.2013 4	**CC005 1:200**	260213-4-1	**4300**			
		260213-4-2				
		260213-4-3				

26.2.2013 5	**CC005 1:200**	260213-5-1	**4132**	**negative (27/06/13) (CC005) negative (27/06/13) (CC010) negative (27/06/13) (CC014) negative (19/07/13) (CC005)**	**/**	**/**
		260213-5-2		**negative (21/06/13) (CC014) negative (21/06/13) (CC005) negative (21/06/13) (CC010)**	**/**	**/**
		260213-5-3				
26.2.2013 6	**CC005 1:200**	260213-6-1	**4040**			
		260213-6-2				
		260213-6-3		**negative (06/07/13) (CC005) negative (06/07/13) (CC010) negative (06/07/13) (CC014) negative (19/07/13) (CC005)**	**/**	**/**
		260213-6-4				
		260213-6-5				
		260213-6-6				
5.3.2013 1	**CC005 1:200**	050313-1-1	**4892**	**negative (22/5/13) (CC005)**	**/**	**/**
5.3.2013 2	**CC005 1:200**	050313-2-1	**4584**	**negative (22/5/13) (CC005)**	**/**	**/**
5.3.2013 3	**CC005 1:200**	050313-3-1	**4132**	**negative (22/5/13) (CC005)**	**/**	**/**
		050313-3-2		**negative (06/07/13) (CC005) negative (06/07/13) (CC014) negative (06/07/13) (CC010) negative (13/07/13) (CC014)**	**/**	**/**
		050313-3-3		**negative (21/03/13) (CC005) negative (14/06/13) (CC005) negative (14/06/13) (CC010)**	**/**	**/**
		050313-3-4		**negative (11/04/13) (CC014)**	**/**	**/**
		050313-3-5		**negative (07/04/13) (CC005) negative (11/04/13) (CC014) negative (21/06/13) (CC010)**	**/**	**/**
		050313-3-6		**negative (11/04/13) (CC014)**	**/**	**/**
5.3.2013 4	**CC014 1:200**	050313-4-1	**4336**	**negative (11/04/13) (CC005)**	**/**	**/**
		050313-4-2		**negative (27/06/13) (CC005) negative (27/06/13) (CC010) negative (27/06/13) (CC014) negative (19/07/13) (CC005)**	**/**	**/**
		050313-4-3		**negative (21/03/13) (CC005) negative (07/04/13) (CC014)**	**/**	**/**
		050313-4-4				
		050313-4-5		**UOB-COL-14 (14/06/13)**	**14/06/13 14/06/13**	**3x(CC005) negative with CC010**

		050313-4-6		negative (01/05/13) (CC005) negative (01/05/13) (CC010) negative (16/06/13) (CC005) negative (14/06/13) (CC010)	/	/
		050313-4-7		negative (21/03/13) (CC005) negative (07/04/13) (CC014)	/	/
		050313-4-8		negative (11/04/13) (CC005) negative (21/06/13) (CC005) negative (21/06/13) (CC010) negative (21/06/13) (CC014)	/	/
5.3.2013 5	**CC014 1:200**	050313-5-1	**5252**			
		050313-5-2		**UOB-COL-15 (14/6/13)**	14/06/13 14/06/13	3x(CC005) negative with CC010
		050313-5-3		**UOB-COL-11 (11/4/13)**	11/04/13	5x(CC005)
		050313-5-4		negative (21/03/13) (CC005) negative (07/04/13) (CC014)	/	/
		050313-5-5		**UOB-COL-3 (14/03/13)**	14/03/13 27/04/13 27/04/13	3x(CC005) 4x(CC005) negative with CC014
5.3.2013 6	**CC014 1:200**	050313-6-1	**4136**	**UOB-COL-4 (14/03/13)**	21/03/13 07/04/13 29/04/13 29/04/13	3x(CC005) 3x(CC014) 4x(CC005) negative with CC014
		050313-6-2				
		050313-6-3		negative (14/03/13) (CC005)	/	/
		050313-6-4		negative (14/03/13) (CC005)	/	/
		050313-6-5				
		050313-6-6		**UOB-COL-6 (14/03/13)**	21/03/13 07/04/13	3x(CC005) 3x(CC014)
		050313-6-7		**UOB-COL-12 (11/4/13)**	11/04/13	5x(CC005)
		050313-6-8		**UOB-COL-5 (21/03/13)**	21/03/13 07/04/13 29/04/13 29/04/13	3x(CC005) negative with CC014 3x(CC014) 5x(CC005)
		050313-6-9		**UOB-COL-10 (11/4/2013)**	11/04/13	5x(CC005)
		050313-6-10		negative (11/04/13) (CC005)	/	/
5.3.2013 7	**CC014 1:200**	050313-7-1	**4976**	**UOB-COL-9 (11/04/13)**	11/04/13	5x(CC005)
		050313-7-2		negative (06/07/13) (CC005) negative (06/07/13) (CC014) negative (06/07/13) (CC010) negative (13/07/13) (CC005)	/	/
		050313-7-3		negative (06/07/13) (CC005) negative (06/07/13) (CC014) negative (06/07/13) (CC010)	/	/
		050313-7-4		negative (14/06/13) (CC005) negative (14/06/13) (CC010)	/	/

		050313-7-5		negative (07/04/13) (CC014)	/	/
				UOB-COL-13 (11/04/13)	11/04/13	5x(CC005)
		050313-7-6		UOB-COL-7 (14/03/13)	21/03/13	3x(CC005)
		050313-7-7				
		050313-7-8		negative (21/06/13) (CC014) negative (21/06/13) (CC005) negative (21/06/13) (CC010) negative (13/07/13) (CC005)	/	/
12.3.2013 1	CC005 1:200	130313-1-1	3856			
12.3.2013 2	CC014 1:200	130313-2-1	4124	negative (21/03/13) (CC014) negative (01/05/13) (CC005) negative (01/05/13) (CC010) negative (14/06/13) (CC005) negative (14/06/13) (CC010)	/	/
		130313-2-2		negative (11/04/13) (CC014) negative (29/04/13) (CC005)	/	/
		130313-2-3		negative (11/04/13) (CC014) negative (29/04/13) (CC005)	/	/
		130313-2-4		negative (11/04/13) (CC014) negative (29/04/13) (CC005)	/	/
		130313-2-5		negative (11/04/13) (CC014) negative (29/04/13) (CC005)	/	/
		130313-2-6		negative (11/04/13) (CC014)	/	/
		130313-2-7		negative (11/04/13) (CC014) negative (29/04/13) (CC005)	/	/
		130313-2-8		negative (11/04/13) (CC014)	/	/
12.3.2013 3	CC014 1:200	130313-3-1	3940	negative (11/04/13) (CC014)	/	/
		130313-3-2		negative (11/04/13) (CC014)	/	/
		130313-3-3		negative (11/04/13) (CC014) negative (29/04/13) (CC005) negative (01/05/13) (CC010)	/	/
		130313-3-4		negative (11/04/13) (CC014) negative (29/04/13) (CC005)	/	/
12.3.2013 4	CC005 1:200	0	4444	/	/	/
19.3.2013 1	CC014 1:200	200313-1-1	4144	negative (15/05/13) (CC014)	/	/
		200313-1-2		negative (21/06/13) (CC014) negative (21/06/13) (CC005) negative (21/06/13) (CC010)	/	/
19.3.2013 2	CC014 1:200	200313-2-1	4552	negative (15/05/13) (CC014)		
19.3.2013 3	CC014 1:200	0	4340	/	/	/
19.3.2013 4	CC014 1:200	200313-4-1	4620	negative (15/05/13) (CC014)	/	/
		200313-4-2		negative (15/05/13) (CC014)	/	/

25.3.2013 1	**CC005 1:200**	0	**3756**	**/**	**/**	**/**
25.3.2013 2	**CC005 1:200**	0	**3564**	**/**	**/**	**/**
25.3.2013 3	**CC005 1:200**	250313-3-1	**4040**	**negative (03/04/13) (CC005)**	**/**	**/**
		250313-3-2		**negative (03/04/13) (CC005)**	**/**	**/**
25.3.2013 4	**CC005 1:200**	0	**3796**	**/**	**/**	**/**
25.3.2013 5	**CC005 1:200**	0	**3844**	**/**	**/**	**/**
25.3.2013 6	**CC014 1:200**	0	**4520**	**/**	**/**	**/**
25.3.2013 7	**CC014 1:200**	0	**4800**	**/**	**/**	**/**
25.3.2013 8	**CC014 1:200**	250313-8-1	**4044**	**negative (03/04/13) (CC014)**	**/**	**/**
		250313-8-2		**negative (03/04/13) (CC014)**		
		250313-8-3		**negative (03/04/13) (CC014)**		
25.3.2013 9	**CC014 1:200**	0	**3852**	**/**	**/**	**/**
25.3.2013 10	**CC014 1:200**	0	**3936**	**/**	**/**	**/**
28.3.2013 1	**CC005 1:200**	0	**4256**	**/**	**/**	**/**
28.3.2013 2	**CC005 1:200**	290313-2-1	**3728**	**negative (03/04/13) (CC005)**	**/**	**/**
		290313-2-2		**negative (03/04/13) (CC005)**	**/**	**/**
28.3.2013 3	**CC005 1:200**	0	**3932**	**/**	**/**	**/**
28.3.2013 4	**CC005 1:200**	0	**4440**	**/**	**/**	**/**
28.3.2013 5	**CC014 1:200**	290313-5-1	**4920**	**negative (03/04/13) (CC014)**	**/**	**/**
28.3.2013 6	**CC014 1:200**	290313-6-1	**4040**	**negative (03/04/13) (CC014)**	**/**	**/**
28.3.2013 7	**CC014 1:200**	0	**3852**	**/**	**/**	**/**
06.4.2013 1	**CC005 1:200**	0	**4440**	**/**	**/**	**/**
06.4.2013 2	**CC014 1:200**	0	**3724**	**/**	**/**	**/**
06.4.2013 3	**CC014 1:200**	0	**4844**	**/**	**/**	**/**
06.4.2013 4	**CC005 1:200**	060413-4-1	**3644**			
		060413-4-2				
		060413-4-3				

		060413-4-4				
06.4.2013 5	**CC005 1:200**	060413-5-1	**3968**			
		060413-5-2				
		060413-5-3				
		060413-5-4				
		060413-5-5				
29.4.2013 1	**CC005 1:200**	0	**3724**	**/**	**/**	**/**
29.4.2013 2	**CC005 1:200**	0	**3460**	**/**	**/**	**/**
29.4.2013 3	**CC005 1:200**	0	**3964**	**/**	**/**	**/**
29.4.2013 4	**CC014 1:200**	0	**2860**	**/**	**/**	**/**
29.4.2013 5	**CC014 1:200**	0	**5520**	**/**	**/**	**/**
29.4.2013 6	**CC014 1:200**	0	**5244**	**/**	**/**	**/**
02.5.2013 1	**CC005 1:200**	0	**4844**	**/**	**/**	**/**
02.5.2013 2	**CC005 1:200**	0	**4524**	**/**	**/**	**/**
02.5.2013 3	**CC005 1:200**	030513-3-1	**4072**	**negative (08/05/13) (CC005)**	**/**	**/**
		030513-3-2		**negative (08/05/13) (CC005)**	**/**	**/**
02.5.2013 4	**CC005 1:200**	0	**4880**	**/**	**/**	**/**
02.5.2013 5	**CC014 1:200**	030513-5-1	**5244**	**negative (08/05/13) (CC010)**	**/**	**/**
02.5.2013 6	**CC014 1:200**	0	**3944**	**/**	**/**	**/**
02.5.2013 7	**CC014 1:200**	0	**4052**	**/**	**/**	**/**
02.5.2013 8	**CC014 1:200**	0	**4660**	**/**	**/**	**/**
02.5.2013 9	**CC010 1:200**	0	**4852**	**/**	**/**	**/**
02.5.2013 10	**CC010 1:200**	0	**4724**	**/**	**/**	**/**
06.5.2013 1	**CC005 1:200**	0	**3892**	**/**	**/**	**/**
06.5.2013 2	**CC005 1:200**	0	**4052**	**/**	**/**	**/**
06.5.2013 3	**CC005 1:200**	0	**4252**	**/**	**/**	**/**
06.5.2013 4	**CC010**	0	**3872**	**/**	**/**	**/**

	1:200					
06.5.2013 5	**CC010 1:200**	0	**4804**	/	/	/
06.5.2013 6	**CC010 1:200**	0	**4644**	/	/	/
13.5.2013 1	**CC005 1:200**	0	**4052**	/	/	/
13.5.2013 2	**CC005 1:200**	0	**4844**	/	/	/
13.5.2013 3	**CC005 1:200**	0	**3964**	/	/	/
13.5.2013 4	**CC005 1:200**	0	**5204**	/	/	/
13.5.2013 5	**CC010 1:200**	0	**3884**	/	/	/
13.5.2013 6	**CC010 1:200**	0	**4344**	/	/	/
13.5.2013 7	**CC010 1:200**	0	**4844**	/	/	/
13.5.2013 8	**CC010 1:200**	0	**5164**	/	/	/
13.5.2013 9	**CC010 1:200**	0	**4644**	/	/	/
13.5.2013 10	**CC010 1:200**	0	**4528**	/	/	/
13.5.2013 11	**CC014 1:200**	0	**3960**	/	/	/
13.5.2013 12	**CC014 1:200**	0	**3936**	/	/	/
13.5.2013 13	**CC014 1:200**	0	**4576**	/	/	/
17.5.2013 1	**CC014 1:200**	0	**4524**	/	/	/
17.5.2013 2	**CC014 1:200**	180513-2-1 180513-2-2 180513-2-3 180513-2-4	**4264**	**negative (22/05/13) (CC014)** **negative (22/05/13) (CC014)** **negative (22/05/13) (CC014)** **negative (22/05/13) (CC014)**	/ / / /	/ / / /
17.5.2013 3	**CC005 1:200**	180513-3-1	**3992**	**negative (29/05/13) (CC005)**	/	/
17.5.2013 4	**CC005 1:200**	0	**4052**	/	/	/
17.5.2013 5	**CC005 1:200**	180513-5-1 180513-5-2 180513-5-3	**3612**	**negative (29/05/13) (CC005)** **negative (29/05/13) (CC005)** **negative (29/05/13) (CC005)**	/ / /	/ / /
17.5.2013 6	**CC005 1:200**	0	**4844**	/	/	/

17.5.2013 7	**CC010 1:200**	0	**5204**	**/**	**/**	**/**
17.5.2013 8	**CC010 1:200**	180513-8-1 180513-8-2 180513-8-3	**5156**	**negative (22/05/13) (CC010)** **negative (22/05/13) (CC010)** **negative (22/05/13) (CC010)**	**/** **/** **/**	**/** **/** **/**
19.5.2103 1	**CC005 1:200**	0	**4480**	**/**	**/**	**/**
19.5.2103 2	**CC005 1:200**	0	**4292**	**/**	**/**	**/**
19.5.2103 3	**CC005 1:200**	0	**4740**	**/**	**/**	**/**
19.5.2103 4	**CC005 1:200**	0	**4852**	**/**	**/**	**/**
19.5.2103 5	**CC010 1:200**	0	**4264**	**/**	**/**	**/**
19.5.2103 6	**CC010 1:200**	0	**4336**	**/**	**/**	**/**
19.5.2103 7	**CC010 1:200**	0	**4852**	**/**	**/**	**/**
19.5.2103 8	**CC010 1:200**	200513-8-1 200513-8-2	**4768**	**negative (29/05/13) (CC010)** **negative (29/05/13) (CC010)**	**/** **/**	**/** **/**
19.5.2103 9	**CC010 1:200**	0	**4440**	**/**	**/**	**/**
19.5.2103 10	**CC014 1:200**	0	**5220**	**/**	**/**	**/**
19.5.2103 11	**CC014 1:200**	0	**5616**	**/**	**/**	**/**
27.5.2013 1	**CC005 1:200**	0	**5464**	**/**	**/**	**/**
27.5.2013 2	**CC005 1:200**	0	**5524**	**/**	**/**	**/**
27.5.2013 3	**CC005 1:200**	0	**5164**	**/**	**/**	**/**
27.5.2013 4	**CC010 1:200**	0	**5644**	**/**	**/**	**/**
27.5.2013 5	**CC010 1:200**	0	**5896**	**/**	**/**	**/**
27.5.2013 6	**CC010 1:200**	0	**5124**	**/**	**/**	**/**
27.5.2013 7	**CC014 1:200**	0	**5252**	**/**	**/**	**/**
27.5.2013 8	**CC014 1:200**	0	**5180**	**/**	**/**	**/**
27.5.2013 9	**CC014 1:200**	0	**4712**	**/**	**/**	**/**
27.5.2013 10	**CC014**	0	**5776**	**/**	**/**	**/**

	1:200					
03.6.2013 1	**CC010 1:200**	0	**4964**	**/**	**/**	**/**
03.6.2013 2	**CC010 1:200**	0	**4704**	**/**	**/**	**/**
03.6.2013 3	**CC010 1:200**	0	**5652**	**/**	**/**	**/**
03.6.2013 4	**CC010 1:200**	0	**5684**	**/**	**/**	**/**
03.6.2013 5	**CC010 1:200**	0	**4692**	**/**	**/**	**/**
03.6.2013 6	**CC005 1:200**	0	**4864**	**/**	**/**	**/**
03.6.2013 7	**CC005 1:200**	0	**5152**	**/**	**/**	**/**
03.6.2013 8	**CC005 1:200**	0	**5432**	**/**	**/**	**/**
03.6.2013 9	**CC014 1:200**	0	**4904**	**/**	**/**	**/**
03.6.2013 10	**CC014 1:200**	0	**4392**	**/**	**/**	**/**
12.6.2013 1	**CC005 1:200**	0	**4736**	**/**	**/**	**/**
12.6.2013 2	**CC005 1:200**	0	**4844**	**/**	**/**	**/**
12.6.2013 3	**CC014 1:200**	0	**4380**	**/**	**/**	**/**
12.6.2013 4	**CC014 1:200**	0	**5204**	**/**	**/**	**/**
24.6.2013 1	**CC005 1:200**	260613-1-1	**5108**			
24.6.2013 2	**CC005 1:200**	260613-2-1 260613-2-2	**5604**	**negative (24/07/13) (CC005)** **negative (24/07/13) (CC005)**	**/** **/**	**/** **/**
24.6.2013 3	**CC005 1:200**	260613-3-1	**5248**	**negative (24/07/13) (CC005)**	**/**	**/**
24.6.2013 4	**CC014 1:200**	0	**5196**	**/**	**/**	**/**
24.6.2013 5	**CC010 1:200**	0	**5444**	**/**	**/**	**/**
24.6.2013 6	**CC010 1:200**	0	**5084**	**/**	**/**	**/**
01.7.2013 1	**CC005 1:200**	0	**5204**	**/**	**/**	**/**
01.7.2013 2	**CC005**	0	**4448**	**/**	**/**	**/**

	1:200					
01.7.2013 3	**CC005 1:200**	030713-3-1 030713-3-2	**4924**	**negative (24/07/13) (CC005)** **negative (24/07/13) (CC005)**	**/** **/**	**/** **/**
01.7.2013 4	**CC005 1:200**	0	**4880**	**/**	**/**	**/**
01.7.2013 5	**CC014 1:200**	030713-5-1	**5244**	**negative (24/07/13) (CC014)**	**/**	**/**
01.7.2013 6	**CC014 1:200**	0	**5288**	**/**	**/**	**/**
01.7.2013 7	**CC014 1:200**	0	**4660**	**/**	**/**	**/**
01.7.2013 8	**CC014 1:200**	0	**5608**	**/**	**/**	**/**
01.7.2013 9	**CC010 1:200**	0	**5244**	**/**	**/**	**/**
01.7.2013 10	**CC010 1:200**	0	**5336**	**/**	**/**	**/**
10.7.2013 1	**CC014 1:200**	0	**4844**	**/**	**/**	**/**
10.7.2013 2	**CC014 1:200**	120713-2-1 120713-2-2	**4852**	**negative (24/07/13) (CC014)** **negative (24/07/13) (CC014)**	**/** **/**	**/** **/**
10.7.2013 3	**CC005 1:200**	0	**5272**	**/**	**/**	**/**
10.7.2013 4	**CC005 1:200**	0	**4224**	**/**	**/**	**/**
10.7.2013 5	**CC005 1:200**	120713-5-1 120713-5-2 120713-5-3	**5068**	**negative (24/07/13) (CC005)** **negative (24/07/13) (CC005)** **negative (24/07/13) (CC005)**	**/** **/** **/**	**/** **/** **/**
10.7.2013 6	**CC005 1:200**	0	**5124**	**/**	**/**	**/**
10.7.2013 7	**CC010 1:200**	0	**5284**	**/**	**/**	**/**
10.7.2013 8	**CC010 1:200**	120713-8-1 120713-8-2 120713-8-3	**4956**	**negative (31/07/13) (CC010)** **negative (31/07/13) (CC010)** **negative (31/07/13) (CC010)**	**/** **/** **/**	**/** **/** **/**
10.7.2013 9	**CC010 1:200**	0	**4884**	**/**	**/**	**/**
10.7.2013 10	**CC010 1:200**	0	**4452**	**/**	**/**	**/**
17.7.2103 1	**CC005 1:200**	0	**4492**	**/**	**/**	**/**
17.7.2103 2	**CC005 1:200**	0	**5248**	**/**	**/**	**/**
17.7.2103 3	**CC005 1:200**	0	**4404**	**/**	**/**	**/**
17.7.2103 4	**CC005**	0	**4348**	**/**	**/**	**/**

	1:200					
17.7.2103 5	**CC010 1:200**	0	**4852**	**/**	**/**	**/**
17.7.2103 6	**CC010 1:200**	0	**4416**	**/**	**/**	**/**
17.7.2103 7	**CC010 1:200**	0	**4816**	**/**	**/**	**/**
17.7.2103 8	**CC010 1:200**	190713-8-1 190713-8-2	**4956**	**negative (31/07/13) (CC010)** **negative (31/07/13) (CC010)**	**/** **/**	**/** **/**
17.7.2103 9	**CC010 1:200**	0	**5204**	**/**	**/**	**/**
17.7.2103 10	**CC014 1:200**	0	**5220**	**/**	**/**	**/**
17.7.2103 11	**CC014 1:200**	0	**5244**	**/**	**/**	**/**